Human placental inositolphosphoglycans may be released by glycosylphosphatidylinositol-phospholipase D, both present in the syncytial brush border membrane.

Investigation in normal and pre-eclamptic placentae

Thesis submitted for the Degree of
Doctor of Philosophy
in the
Faculty of Science
at the
University College London
by
Sylvie Deborde

Molecular Medicine Unit,
Department of Molecular Pathology,
University College London.

September 2000
ABSTRACT

Pre-eclampsia is a disorder affecting 5 to 10% of all pregnancies. In the pre-eclamptic placenta, increased levels of an inositolphosphoglycan (IPG) second messengers have been reported. The objective of this thesis has been the study of IPGs in the placenta and their possible release by the glycosylphosphatidylinositol-phospholipase D (GPI-PLD) enzyme.

The presence of IPGs was investigated in the placenta. Immunostaining on sections of chorionic villi revealed the presence of IPGs and confirmed the higher levels of IPGs in pre-eclampsia. IPGs of both sub-types A and P were extracted from microvilli preparations of both normal and pre-eclamptic placentae.

After showing the presence of biologically active IPGs in microvilli preparation, the next step was to investigate the presence of glycosylphosphatidylinositol (GPI), the putative precursor molecule of IPGs. GPI was extracted from microvilli of normal placenta but unexpectedly not detected in pre-eclamptic samples.

An elevated GPI-PLD activity could cause increased catabolism of GPI, and thus be responsible for both the absence of detectable GPI and the increased IPG levels found in pre-eclamptic samples. GPI-PLD hydrolysis activity was measured in microvilli preparations from both normal and pre-eclamptic placenta.

Whereas GPI-PLD is described as a 100 kDa protein, a protein of 50 kDa was mainly detected. GPI-PLD mRNA was not detected in the placenta. We therefore propose a model in which placental GPI-PLD is taken up by the syncytiotrophoblast from the maternal blood and transferred to a lysosomal compartment where it is cleaved into an active 50 kDa protein before returning to the plasma membrane.

Finally, the susceptibility of extracted placental GPI to hydrolysis by recombinant GPI-PLD has been studied. GPI-PLD was able to cleave GPI in vitro.

To conclude, this work confirms the presence of IPGs in placenta and more specifically in microvilli and supports previous reports of increased IPG-P type in pre-eclampsia. The study of placental GPI and GPI-PLD throws light on the possible mechanism underlying the generation of IPGs in both normal and pre-eclamptic microvilli.
ACKNOWLEDGEMENTS

I first want to acknowledge the person who introduced me to the world of IPGs, my supervisor Professor Thomas Rademacher, for giving me the opportunity to study in this laboratory and for his enthusiasm.

I also like to thank Julian Schofield, for his generous help in practical work as well as in thesis writing. He provided the recombinant GPI-PLD, taught me PCR and was a very good sounding board.

I am indebted to Sirilaksana (Betty) Kunjara, Lance Turtle, Keiju Puan and Dev Sooranna for their kindness and practical assistance. Betty performed the puruvate dehydrogenase phosphatase activity assays and was always ready to help in the laboratory. Lance was very helpful in the work concerning the GPI investigation. Keiju provided radiolabelled mfVSG and helped to measure its hydrolysis. Dev gave me BeWo cells, taught me the immunocyto-chemistry work, and introduced me to placentologists.

Professor Avrion Mitchison and Dr Lawrence Pitkethly are two persons I also want to thank for their proof reading and comments.

I also like to acknowledge all my colleagues of the laboratory of the molecular medicine unit and of RGL company (Phil, Hugo, Alan, Richard, Bia, Stephane, Antonio) for their kindness and help.

I am also grateful to RGL company for the financial support.

My sister, Céline has also a place in this page. I like to acknowledge her for her exceptional ability to transmit joy and support through telephone.

And of course, I can not forget to be grateful to my boyfriend, Pascal, who put up with me during this thesis.
# TABLE OF CONTENTS

| TITLE PAGE | 1 |
| ABSTRACT | 2 |
| ACKNOWLEDGEMENTS | 3 |
| TABLE OF CONTENTS | 4 |
| ABBREVIATIONS | 9 |
| LIST OF FIGURES | 11 |
| LIST OF TABLES | 14 |

## GENERAL INTRODUCTION- PRE-ECLAMPSIA

- Preeclampsia 16
- The placenta in pre-eclampsia 16
- Placentaion in pre-eclamptic pregnancy 17
- The syncytial microvillus membrane 18
- Microvilli are tubualr caveolae 19
- Normal and pre-eclamptic placenta: structural features 19
- Shedding of microvilli 20
- Normal and pre-eclamptic placenta: biochemical features 20
- Inositolphosphoglycans and pre-eclampsia 21
- Leptin and pre-eclampsia 21
- BeWo trophoblast cell line 22
- Hypotheses 22
- Aim and structure of the thesis 22

## CHAPTER 1 – IPGs IN PLACENTA

### INTRODUCTION

- Introduction 32
- IPGs discovery 32
- Further studies: chemical characteristics, release by PI-PLC, a glycolipid precursor 33
- Two subfamilies: IPG A- and P-type 35
- IPGs mimic insulin and other factors 36
- IPGs in signal transduction 37
- Generation of IPG 39
- IPG cellular location 40
- IPGs in placenta: presence and function 40
- IPGs and pathological conditions 40
- Reasons for investigating IPG in the normal and pre-eclamptic placenta 42
- Aim of the chapter 42

### MATERIALS & METHODS

- Immunohistochemistry 46
- Syncytial microvillus membrane preparations 47
Preparation of the microvillous membrane 47
Protein assay 48
Alkaline phosphatase assay 49
IPGs extraction procedure 50
Measurement of IPG A-type and IPG P-type activity 51
Cell proliferation assay 51
cAMP dependent protein kinase A assay 53
Pyruvate dehydrogenase phosphatase activity assay 54
Measurement of the effect of IPGs on the release of leptin from BeWo cell 55
   BeWo cell culture 56
   Measurement of leptin secretion 56
Measurement of the effect of IPGs on the proliferation of BeWo cells 58

RESULTS 59

IPG Immunocytochemistry on chorionic villi sections of normal and pre-eclamptic placentae 59
Presence of IPG activity in both homogenates of placenta and in microvilli preparations 59
   Cell proliferation assay 59
cAMP dependent protein kinase A assay 60
   PDH phosphatase assay 60
IPGs and proliferation of BeWo cells 61
Active placental IPGs did not modulate leptin release from BeWo cells 61

DISCUSSION 73

Microvillous IPG A- and P-type 73
IPGs and leptin release 74
IPGs location in the placenta 75
Caveolae 78
IPGs and signal transduction in the placenta 78
IPGs and pre-eclampsia 80
Pre-eclampsia: a defect of IPG/GPI signal transduction? 81
Summary 82

CHAPTER 2 - GPI IN PLACENTA 84

INTRODUCTION 85

Structure of the IPG precursor molecules 85
   The polar head group 85
   The lipid moiety 85
IPG precursor related molecule: free GPI of mammalian cells 86
IPG precursor related molecules: GPI-anchored proteins 86
IPG precursor related molecules: GIPLs, LPGs and LPPGs 89
GPI cellular location 90
Reasons for investigating GPI in the normal and pre-eclamptic placenta 91
Aim of the chapter 92
MATERIALS & METHODS

GPI extraction 96
Determination of phosphate content 98
Determination of amino-group content 98

RESULTS 101

Isolation of GPI from total placental membrane of normal placentae 101
Isolation of GPI from microvillous membrane of normal placentae 101
Absence or not detectable amount of GPI in pre-eclamptic placentae 102
Amino-group and phosphate contents of the GPI preparation 102

DISCUSSION 111

Findings 111
Presence of GPI in microvilli 112
Absence or low level of GPI in pre-eclamptic placenta 112
Glycolipid composition of microvilli 113
Conclusion 114

CHAPTER 3 - GPI-PLD IN PLACENTA 116

INTRODUCTION 117

What is GPI-PLD? 117
Discovery of GPI-PLD activity 118
Purification of the protein 118
GPI-PLD mRNA expression 119
Regulation of the activity 120
Functional role 122
Tissue expression 123
Cellular localisation 124
Reasons for investigating GPI-PLD in the placenta 125
Aim of the chapter 125

MATERIALS & METHODS 127

Immunohistochemistry 127
Western-blot 128
GPI-PLD activity assay using mfVSG 129
GPI-degradation assay 129
Identification of products of GPI-anchor converting activity by TLC analysis of mfVSG 130
GPI-PLD mRNA investigation 130
RNA extraction 131
cDNA synthesis 131
RT-PCR 132
RESULTS

Evidence of GPI-PLD protein and GPI-PLD activity in the placenta, but no detectable GPI-PLD mRNA

DISCUSSION

GPI-PLD, a 50 kDa protein present in the human placenta
Presence of GPI-PLD in the syncytial microvillous membrane
Demonstration of GPI-PLD activity and not GPI-PLC activity
GPI-PLD activity in normal and pre-eclamptic placenta
The placenta does not synthesise GPI-PLD
Model of uptake of GPI-PLD

CHAPTER 4 – HYDROLYSIS OF GPI BY GPI-PLD

INTRODUCTION

IPGs in the Placenta
GPI in the Placenta
GPI-PLD in the Placenta
GPI-PLD in the IPG/GPI Cellular Signalling System
Hypotheses
Aim of the Chapter

MATERIALS & METHODS

Detection of GPI after GPI-PLD treatment
Detection of lipidic products of the reaction
Measure of IPG-like activity

RESULTS

Disappearance of GPI after GPI-PLD treatment
Detection of DAG after placental GPI treatment with GPI-PLD
Generation and assessment of an IPG-like activity from extracted placental GPI treated with GPI-PLD

DISCUSSION

Findings
Two methods
Possible modulation of GPI-PLD activity by phosphatidylcholine / phosphatidylethanolamine (PE/PC) and detergent
IPG/GPI signal transduction in the placenta
Summary
ABBREVIATIONS

ATP, adenosine tri-phosphate
bFGF, basic fibroblast growth factor
BSA, bovine serum albumine
cAMP, cyclic adenosine mono-phosphate
cDNA, complementary DNA
cpm, counts per minute
CRD, cryptic carbohydrate
DAF, decay accelerating factor
DAG, diacetylglycerol
DNA, deoxyribonucleic acid
DTT, dithiothreitol
EDTA, ethylenediaminetetraacetic acid
EGTA, ethyleneglycoltetraacetic acid
ELISA, enzyme-linked immunosorbent assay
GPI, glycosylphosphatidylinositol
GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D
HPTLC, high performance thin layer chromatography
IPG, inositolphosphoglycan
mfVSG, membrane form variant surface glycoprotein
NAD, nicotinamide adenine dinucleotide, oxidised form
NADH, nicotinamide adenine dinucleotide, reduced form
NaF, sodium fluoride
NGS, normal goat serum
PA, phosphatidic acid
PDC, pyruvate dehydrogenase complex
PDH, pyruvate dehydrogenase
PKA, protein kinase A
PLD, phospholipase D
PMSF, phenylmethylsulfonylfluoride
Rf, resolution front
RIA, radioimmunoassay
rGPI-PLD, recombinant GPI-PLD
RNA, ribonucleic acid
TLC, thin layer chromatography
TPP, thiamine pyrophosphate
LIST OF FIGURES

GENERAL INTRODUCTION

Figure 1: Trophoblast differentiation at the implantation site in normal pregnancy 24
Figure 2: Diagram of a longitudinal section of an anchoring chorionic villus at the fetal-maternal interface at ~ 10 week gestational age 26
Figure 3: Diagram of a spiral artery in which endovascular invasion is in progress 27
Figure 4: Schematic drawing of the basic villous morphology 28
Figure 5: Transmission electron micrograph of sections through the cell surface of the syncytiotrophoblast 29
Figure 6: Factors regulating placental glycogen metabolism in pre-eclampsia and diabetes 30

CHAPTER 1

Figure 1.1 : Simple model of IPG release 43
Figure 1.2 : Immunohistochemical staining of IPG in villi sections of normal and pre-eclamptic placenta 65
Figure 1.3 : Incorporation of [3H]thymidine induced by serial dilutions of IPG A-type and IPG P type extracted from intact placenta 66
Figure 1.4 : Incorporation of [3H]thymidine induced by serial dilutions of IPG A-type and IPG P-type extracted from microvilli preparation of normal placenta 67
Figure 1.5 : Incorporation of [3H]thymidine induced by serial dilutions of IPG P-type extracted from microvilli preparations of a normal placenta and a pre-eclamptic placenta 68
Figure 1.6 : Inhibition of cAMP-dependent protein kinase A induced by increased amount of IPG A type 69
Figure 1.7 : Stimulation of pyruvate dehydrogenase phosphatase induced by extracts of IPG P type from normal and pre-eclamptic microvillous membrane preparations 70
Figure 1.8 : Incorporation of [3H]thymidine into BeWo cells in the presence of serial dilutions of IPG A type and IPG P type 71
Figure 1.9 : Determination of leptin released by BeWo cells 72
Figure 1.10: Hypothetical model of IPG release in the microvillous membrane of normal and pre-eclamptic placenta 83

CHAPTER 2

Figure 2.1 : Structures of H6, H7 and H8 93
Figure 2.2 : Structures of known mammalian GPI anchors 94
Figure 2.3 : Proposed divergent biosynthetic pathways for protein-bound and free GPs (IPG-precursors) 95
Figure 2.4 : Placental GPI isolation
Figure 2.5 : Scheme representing the conversion of a non-fluorescent fluo-
rescamine into a fluorescent fluorescamine form by reaction
with primary amino groups
Figure 2.6 : TLC plates of GPI extracts from total plasma membrane of a
normal and a pre-eclamptic placenta
Figure 2.7 : TLC of lipid extracted from total plasma membrane of normal
placenta, detected by iodine and anisaldehyde staining
Figure 2.8 : TLC of GPI extracted from total plasma membrane and micro-
villous membrane
Figure 2.9 : Determination of amino group content in GPI
Figure 2.10: Absorbance of the phosphate standard used for determination
of phosphate in GPI and absorbance of the phosphate stan-
dard added to GPI
Figure 2.11: Linear dose relationship between phosphate and GPI
Figure 2.12: Phosphate absorbance measurements in known amounts of
phosphatidylinositol and inorganic phosphate solutions
Figure 2.13: Hypothetical model of IPG release in the microvillous mem-
brane of normal and pre-eclamptic placenta

CHAPTER 3

Figure 3.1 : Scheme indicating the site of cleavage of phospholipase D on
phospholipid molecules
Figure 3.2 : Immunohistochemical staining of GPI-PLD in a villi section
of a normal human term placenta
Figure 3.3 : Western-blotting of GPI-PLD in homogenate of placenta and
microvilli of normal and pre-eclamptic placentae
Figure 3.4 : Hydrolysis of mfVSG induced by serial dilution of placental
Microvilli
Figure 3.5 : Time-dependent hydrolysis of mfVSG by placental microvilli
Figure 3.6 : Scheme indicating the lipidic products obtained after hydrolys-
is by different phospholipases.
Figure 3.7 : Autoradiograph of a TLC plate demonstrating the nature of the
mfVSG hydrolysis induced by placental microvillous mem-
brane
Figure 3.8 : RT-PCR for β-actin and GPI-PLD in normal and pre-eclamptic
Placentae
Figure 3.9 : Model for GPI-PLD uptake explaining the presence of a 50
kDa GPI-PLD form in the syncytiotrophoblast microvillous
membrane
Figure 3.10: Hypothetical role of GPI-PLD in the model of IPG release in
the microvillous membrane of normal and pre-eclamptic pla-
centa

CHAPTER 4

Figure 4.1: Representation of HPTLC of GPI after GPI-PLD treatment
Figure 4.2: TLC of $[^{32}\text{P}]\text{PA}$ for detection of DAG after placental GPI incubation with GPI-PLD in the presence of alkaline phosphatase 161

Figure 4.3: IPG-like activity in the reaction mixture after GPI hydrolysis by GPI-PLD 162

Figure 4.4: Measure of cAMP-dependent PKA inhibition induced by water phases obtained by fractionation of samples in which GPI has been incubated with GPI-PLD for hydrolysis 163

Figure 4.5: Measure of IPG-like activities on both aqueous and organic phases after butanol fractionation of samples in which GPI has been incubated with GPI-PLD 164
LIST OF TABLES

CHAPTER I

Table 1.1: Insulin-like effects of IPGs 44
Table 1.2: Regulation of GPI hydrolysis 45
Table 1.3: Activities of IPGs obtained from different extractions 63
Table 1.4: Decrease of IPG activity within one week 64
General Introduction

Pre-eclampsia
GENERAL INTRODUCTION

Pre-eclampsia

Pre-eclampsia, an important cause of fetal and maternal perinatal morbidity or mortality, is a specific disorder of human pregnancy, which affects 5 to 10% of all pregnancies. It is characterised by high blood pressure, proteinuria, oedema, abnormal blood clotting and in severe cases, liver and kidney damage. Not all of these features may occur, however, in all instances of pre-eclampsia; and because of the extreme variation between cases there has been considerable argument about definitions. What is common to the disorder is that there is always high blood pressure accompanied by proteinuria. The classification of hypertensive disorders of pregnancy established by the national high blood pressure education program working group recognised pre-eclampsia and eclampsia as a disease with increased blood pressure accompanied by proteinuria (> 300 mg / 24 hour specimen), oedema or both, occurring almost exclusively after 20 weeks' amenorrhoea. Increased blood pressure is defined as a rise in pressure of ≥ 15 mmHg diastolic and by ≥ 30 mmHg systolic from when it was measured in early pregnancy or to ≥ 140/90 mmHg in late pregnancy if prior blood pressure is not known (National High Blood Pressure Education Program 1990).

Eclampsia is a more severe version of pre-eclampsia, which includes all the symptoms of pre-eclampsia but in addition is characterised by grand mal-seizures. Whereas it is accepted that the maternal symptoms result from widespread alterations in endothelial function (Roberts et al 1989), the aetiology of pre-eclampsia is still poorly understood.

The placenta in pre-eclampsia

Compelling evidence implicates the placenta in the disease (Redman 1991). Firstly, delivery cures the disease and secondly some cases of hydatidiform moles are capable of producing pre-eclampsia like symptoms (Hydatidiform moles are types of pregnancy in which the conceptus consists of placental tissue but lacks a fetus). It is clear that the major problem in pre-eclampsia is an insufficiency of the uteroplacental circulatory system, which is thought to be due to a defect of placentation occurring many months before the disorder manifests itself (Redman
This defect of placentation is due to a defect of trophoblastic invasion of the spiral arteries (Brosens et al 1972), a process which happens in normal pregnancy. The placentation in normal pregnancy is described in the legend of figures 1, 2 and 3. Figure 1 is a diagram of the trophoblast differentiation at the implantation site in normal pregnancy, Figure 2 is a diagram of a longitudinal section of an anchoring chorionic villus at the fetal maternal interface at ~ 10 week gestational age and Figure 3 is a diagram of spiral artery in which endovascular invasion is in progress (10-18 week gestation).

Placentation in pre-eclamptic pregnancy

In pre-eclampsia, the affected part of the placenta is the portion which attaches to the uterine wall (Brosens et al 1972, Gerretsen et al 1981, Khong et al 1986, Zhou et al 1993). The arteries are not remodelled as in normal pregnancy, resulting in a poor uteroplacental circulation and a lack of blood supply. This defect is due to a failure of trophoblast invasion (Brosens et al 1972). The endovascular invasion is seriously affected; it does not expand beyond the superficial portions of the uterine spiral arterioles. Thus, these pre-eclamptic vessels are similar to vessels from non-pregnant women: they retain their endothelial lining and muscular walls resulting in narrow-bore, high resistance vessels.

In normal pregnancy, during the invasive stage cytotrophoblast cells differentiate and upregulate the expression of molecules such as integrin α1 (Damsky et al 1992) and metalloproteinase-9 (Librach et al 1991). In pre-eclampsia, this upregulation fails (Genbacev et al 1996, Redline and Patterson 1995). By detecting epithelial and endothelial type adhesion molecules on invading cytotrophoblast, Zhou et al (Zhou et al 1997a) suggest that the invasive differentiating cytotrophoblast cells change their adhesion phenotype from one that is characteristic of epithelial cells to one that is characteristic of vascular cells. In pre-eclampsia, the usual adhesion phenotype of invading cytotrophoblast does not seem to be present suggesting that the adhesion phenotype switch does not happen (Zhou et al 1997b). This switch has been proposed to be necessary for successful endovascular invasion and, more generally, normal placentation.
The syncytial microvillous membrane

The human placenta is a very heterogeneous organ characterised by complex anatomical interactions between fetal and maternal tissues. In the human hemomonochorial placenta, the villi, in direct contact with the maternal blood, form the main functional structure. As shown in the schematic drawings (Figure 4) a villus consists of trophoblast surrounding a core of connective tissue including fetal vessels, fibroblasts and Hofbauer cells (macrophages). At term, the trophoblast comprises an incomplete layer of mononucleated cytotrophoblasts and an uninterrupted layer of multinucleated syncytiotrophoblast which is actually a syncytium of cytotrophoblasts, the result of the interaction, aggregation and fusion of cytotrophoblastic cells. The syncytiotrophoblast, considered as the prime candidate for numerous placental functions, is in contact with the maternal blood by microvilli which form a brush border plasma membrane. This microvillous membrane, which forms the surface of contact between the placenta and the maternal blood, can be easily isolated from the rest of the placenta using a method first described in 1974 (Smith et al 1974). The method was then used and modified by several groups (Arkwright et al 1993, Eaton and Oakey 1994, Grassl 1996, Kaczan-Bourgois et al 1996, Meredith and Laynes 1996, Grassl 1998, Reboucet et al 1998, Lafond et al 1999). In this thesis, preparations of microvillous membrane were extensively studied. Studies have been done on the protein components of the microvillous membrane. Many enzymatic, receptor and transport activities were reported to be present. Many of the proteins of the microvilli detected by SDS-PAGE (Carlson et al 1976, Okamura et al 1981) have not been yet identified. GPI-anchored proteins, proteins characterised by a linkage to the plasma membrane via a covalent binding to a lipid tail, are present in high levels in the apical pole membrane of epithelial cells such as the microvillous membrane of the syncytiotrophoblast. Alkaline phosphatase is one of these numerous GPI-anchored proteins and is used as a marker of the microvillous membrane. Reports on the lipidic composition of the microvillous membrane of the syncytiotrophoblast are also available indicating its richness in cholesterol, sphingolipid and phospholipid (Smith and Brush 1978, Lafond et al 1993, Ghosh and Mukherjea 1995).
Microvilli are tubular caveolae

Placental microvilli were designated as tubular caveolae by Anderson (Anderson 1998). Caveolae are usually described in the literature as invaginations of the plasma membrane with different biochemical composition and physical properties compared to the rest of the plasma membrane. Following the morphological criteria, caveolae were observed in the syncytial brush border membrane of monkey (Enders et al 1983) and human placenta (Ockleford and Whyte 1977). An electron micrograph from this later study is shown in Figure 5. Caveolae were defined by the authors as invaginations localised at the base of the microvilli. These studies were done on first trimester placentae, and raised the question of the presence of such organelles in term placentae. The presence of caveolae is not clearly mentioned in syncytial microvilli of term placenta by placentologists. In contrast, Anderson, a caveolae specialist designates the microvilli as tubular caveolae of placental epithelium. According to him, caveolae, which assume a variety of shapes, including flat, vesicular and tubular, are defined according to their biochemical and physical properties. They have to be resistant to solubilisation by the detergent Triton X-100 at 4°C, they have a light buoyant density and they are rich in sphingolipids, cholesterol and lipid-anchored membrane proteins. Therefore, we assume that the caveolae, as described by Ockleford and Enders, are the one extreme part of caveolae in Anderson’s definition. This idea that microvilli and caveolae are not two but one organelle is supported by the co-localisation of the two structures.

Normal and pre-eclamptic placenta: structural features

Although the appearance and histopathology of pre-eclamptic placentae are variable, pre-eclamptic placentae are often smaller due to a reduced mass of syncytiotrophoblast (Boyd et al 1983). Structural and biochemical features of normal and pre-eclamptic placentae have been compared. A detailed report of the ultrastructure and ultrahistochemistry of pre-eclamptic placentae (Jones and Fox 1980) shows focal necrosis of syncytiotrophoblast with loss and distortion of microvilli with thickened bulbous tips forming an abnormal club-like shape, dilatation of syncytial rough endoplasmic reticulum, decreased syncytial pinocytotic activity and a reduced number of syncytial secretory droplets. The cytotrophoblast is
hyperplasic, there is a degeneration of occasional cytotrophoblastic cells and thickening of the trophoblastic basement membranes.

*Shedding of microvilli*

During pregnancy, trophoblast deportation and syncytial microvilli shedding into the maternal circulation occurs. This process is emphasised in pre-eclampsia (Redman 1993, Knight et al 1998).

It has been observed that microvilli inhibit the proliferation of endothelial cells *in vitro*, microvilli components may therefore account for the endothelial dysfunction characterising the pre-eclamptic disorder (Kertesz et al 1999). It is interesting to note that a similar phenomenon of shedding of plasma membrane fragments also occurs in the liver of patients with cholestatis (De Broe et al 1975). Cholestatis is a disease with an increased expression of alkaline phosphatase at the surface of hepatocyte plasma membrane (Desmet 1992).

*Normal and pre-eclamptic placenta: biochemical features*

In pre-eclamptic placentae, there is reduced alkaline phosphatase activity and dehydrogenase activity in syncytiotrophoblast but increased acid phosphatase activity compared to normal placentae (Jones and Fox 1976, Jones and Fox 1980). Interestingly, whereas alkaline phosphatase activity is generally decreased specifically in microvilli and distributed throughout the trophoblast (Jones and Fox 1976, Jones and Fox 1980), it is increased in the circulation of the pregnant pre-eclamptic woman (Benster 1970, Adeniyi and Olatunbosun 1984). The reason for this has not been elucidated. It could be due to amplified syncytial microvilli shedding or a mechanism which prevents the protein from being anchored properly in the membrane (This last concept will be mentioned in the chapter 2 of this thesis). Numerous other biochemical changes in the placenta are associated with pre-eclampsia, they are well reviewed by Redman (Redman 1993). To this long list can be added, the insulin mediator IPG P-type (Kunjara et al 2000a), the circulating hormone, leptin (Mise et al 1998) and the neurotransmitter, neurokinin B (Page et al 2000).
Inositolphosphoglycans (IPGs) and pre-eclampsia

IPGs are second messenger compounds and are the subject of Chapter 1 of this thesis. Our group extracted IPG P-type (P because it stimulates PDH phosphatase) and IPG A-type (A because it regulates levels of cAMP) from human urine and placenta (Kunjara et al 2000a). Placental IPG P activity was 3 fold higher in pre-eclamptic placenta and urine samples compared to samples from matched normal pregnant women.

Pre-eclamptic placenta accumulates an abnormal amount of glycogen (Arkwright et al 1993). The glycogen content was found to be 10 fold higher in pre-eclamptic placentae than in control placentae matched for gestational age at delivery and this higher glycogen content was associated with 16 times more glycogen synthase and 3 times more glycogen phosphorylase activity. Excess glycogen in the placenta is not only found in pre-eclampsia but also in diabetes. As illustrated in Figure 6 (from Kunjara et al 2000a), the excess of glycogen in diabetic placentae is caused by a higher level of glucose-6 phosphate and circulating glucose, which results in stimulation of glycogen synthase. In pre-eclampsia, the level of circulating glucose is normal. The excess glycogen content in the placenta is thought to be caused by the increase in IPG P-type activity which stimulates the glycogen synthase phosphatase enzyme, which in turn converts an inactive phosphorylated glycogen synthase into an active dephosphorylated form (Kunjara et al 2000a).

Leptin and pre-eclampsia

Leptin is a 167 amino acid circulating protein, involved in the regulation of energy homeostasis, and the neuroendocrine and reproductive system; it is secreted by both adipose tissue (Zhang et al 1994) and the placenta (Masuzaki et al 1997).

In humans, plasma leptin levels are higher in pregnant women than in non-pregnant women matched for age (Masuzaki et al 1997). After delivery, the level decreases to normal values suggesting that the placenta may be the source of the increased circulating leptin during pregnancy. In pre-eclampsia, levels of circulating leptin were found to rise due to higher production by the placenta (Mise et al 1998).

Interestingly, leptin and IPGs, which are both upregulated in pre-eclampsia, were found to be connected in rat adipocytes. The release of leptin was modulated by insulin and IPGs. IPG A-type, but not IPG P-type, had an inhibitory effect,
contrasting with insulin which stimulated leptin release (Kunjara et al. 2000b). In pre-eclamptic placentae it was IPG P-type and not IPG A-type activity which was found to be higher compared to normal placentae (Kunjara et al. 2000a). To date, there is no information about a possible regulation of placental leptin by IPGs in normal and pre-eclamptic pregnancy.

**BeWo trophoblast cell line**
The same group, that showed that the placenta produces leptin, also showed that BeWo cells produce leptin (Masuzaki et al. 1997). Interestingly, when cultured under hypoxic conditions, leptin production was elevated, thus mimicking the pre-eclamptic condition. BeWo cells, a trophoblast cell line generated from choriocarcinoma cells (Pattillo et al. 1968, King et al. 2000) are a powerful tool for use in the understanding of trophoblast cell biology and endocrine function. These cells were cultured in our laboratory and were used in the present investigation.

**Hypotheses**
The present study follows on from the work of Kunjara et al. (Kunjara et al. 2000a) in which higher activity of the IPG P-type was seen in pre-eclamptic urine and placenta. Because IPGs may have been generated from the the caveolae of adipocytes (Parpal et al. 1995), we suspected their presence in syncytial microvilli. We hypothesised that IPGs associated with shed microvilli maybe a candidate for the circulating placental factor X, responsible for the pre-eclamptic disorder. Because leptin is produced by the placenta and its release is inhibited by IPG A-type, we wanted to know if increased IPG A-type or IPG P-type might be responsible for modulation of leptin levels in placenta. We investigated this in vitro using cultured BeWo cells. Finally, we hypothesised a defect in IPG/GPI signalling system which could explain the higher levels of IPG P-type in pre-eclampsia. We therefore also investigated the IPG precursor molecule and the enzyme responsible for IPG release.

**Aim and structure of the thesis**
Considering IPGs as the possible factor responsible for the pre-eclamptic disorder, the goal of this thesis has been to investigate the presence of IPGs in the placenta and study their mechanism of release from microvilli, the syncytial plasma membrane facing the maternal blood. In the first chapter, IPGs were located in the placental
villus. After showing the presence of IPGs in microvilli preparations, the presence of their precursor molecule, glycosylphosphatidylinositol (GPI), was investigated (second chapter). The third chapter concerns glycosylphosphatidylinositol-phospholipase D (GPI-PLD), the enzyme thought to be responsible for IPG release. Finally, in chapter four, the hydrolysis of extracted placental GPI by recombinant GPI-PLD was studied.

List of Hypotheses of the thesis:
IPG/GPI signalling system is present in the human placenta and is defective in pre-eclampsia.

Chapter 1:  IPGs are associated with microvilli
  IPGs regulate placental leptin levels
Chapter 2:  GPI is present in the human placenta
  GPI is present in microvilli
  Level of GPI is higher in pre-eclamptic placentae
Chapter 3:  GPI-PLD is present in microvilli
  GPI-PLD activity differs in normal and pre-eclamptic placentae
Chapter 4:  IPG is released from placental GPI after GPI-PLD treatment
Figure 1: Trophoblast differentiation at the implantation site in normal pregnancy.
The formation of the placenta begins during implantation, on the 6th-7th day after ovulation, when the blastocyst penetrates the maternal endometrium to become embedded in the uterine stroma. The trophoderm cells, which form a layer encircling the blastocyst, differentiate into a syncytial mass at the site of attachment. The syncytial mass, which must result from differentiation of underlying mononuclear cytotrophoblast cells as mitosis is not seen in syncytiotrophoblast, penetrates between epithelial cells and rapidly expands into the underlying stroma. In the syncytium, vacuoles or lacunae form, enlarge and fuse to communicate with each other. A potential uteroplacental circulation is established when maternal venous capillaries are eroded by the syncytium so that blood can enter the trophoblastic lacunae. By 12-13 days after fertilisation, the blastocyst is completely embedded in the decidual stroma and chorionic villi form in the lacunae. During the second week of gestation, buds of cytotrophoblast push into the primitive syncytium to form the primary villi. Another tissue, mesoderm, then emerges in the trophoblast to form the secondary villi. The third stage is when blood vessels develop soon after in the mesodermal cores of the villi. Thus, by the fourth week of pregnancy, the circulatory
system threading the foetus and placenta is in place. The villi spread and segment swiftly so that by the second and third months of pregnancy the villous tree characteristic of mature placenta is established. Whereas the majority of the villi float free in the pool of maternal blood, some of them are attached to the decidua. After the second week, at the tips of the early villi, cytotrophoblast cells form cell columns which fix the villi to the decidua, forming anchoring villi. The cytotrophoblast cell columns spread laterally and fuse with neighbouring columns to form a cytotrophoblast shell which encircles the entire embryonic sac. Interstitial and endovascular invading trophoblast cells arise from this shell. Interstitial trophoblast cells invade the uterine mucosa and the decidua where they are found concentrated around the arteries. They seem to have a specific and remarkable effect on the spiral arteries since decidual basalis arteries, which are surrounded by trophoblast show endothelial swelling and a characteristic destruction of the muscular media which is replaced with ‘fibrinoid’ material. By 8 weeks of pregnancy interstitial trophoblast has extensively colonised the full thickness of the uterine mucosa to reach the decidual-myometrial border. Deeper into the decidua, the trophoblast cells become multinucleated and more rounded, they are known as placental bed giant cells. During the second trimester, there is further invasion into the inner myometrium. Destruction of the musculo-elastic tissue of the intramyometrial segments of the spiral arteries occurs with similar fibrinoid change of the media. The cytotrophoblast shell provides not only interstitial invading trophoblast cells, but also endovascular invading trophoblast cells. Trophoblasts enter the arteries where they move in a retrograde manner down the spiral arteries and replace the maternal endothelium on the vessel walls. Extension of endovascular trophoblast continues into the arteries throughout the first trimester and invasion expands as far as the first third of the myometrium. The endovascular trophoblast invasion is associated with degeneration of the vascular endothelium and the loss of musculo-elastic tissue previously described, converting the maternal spiral arteries into large tubes with perivascular trophoblast embedded in the vessel wall. This results in the spiral arteries becoming larger to ensure an increased maternal blood flow necessary for the growing placenta (Adapted from (Loke and King 1996)).
**Figure 2**: Diagram of a longitudinal section of an anchoring chorionic villus (AV) at the fetal-maternal interface at ~ 10 week gestational age. The anchoring villus (AV) functions as a bridge between the fetal and maternal compartments, whereas floating villi (FV) are suspended in the intervillus space and are bathed by maternal blood. Cytotrophoblast in AV (Zone I) form cell columns (Zones II & III). Cytotrophoblast then invade the uterine interstitium (decidua and first third of the myometrium, (Zone IV) and maternal vasculature (Zone V), thereby anchoring the fetus to the mother and accessing the maternal circulation (From reference (Zhou et al 1997a)).
Figure 3: Diagram of a spiral artery in which endovascular invasion is in progress (10-18 week gestation). Endometrial and then myometrial segments of spiral arteries are modified progressively. In fully modified regions (a) the vessel diameter is large. Cytotrophoblasts are present in the lumen and occupy the entire surface of the vessel wall. A discrete muscular layer (tunica media) is not evident. (b) Partially modified vessel segments. Cytotrophoblasts and maternal endothelium occupy discrete regions of the vessel wall. In areas of intersection, cytotrophoblasts appear to lie deep to the endothelium and in contact with the vessel wall. (c) Unmodified vessel segments in the myometrium. Vessel segments in the superficial third of the myometrium will become modified when endovascular invasion reaches its fullest extent (by 22 week), while deeper segments of the same artery will retain their normal structure. (From reference (Zhou et al 1997a)).
Figure 4: Schematic drawing of the basic villous morphology. The chorionic villus is covered by the trophoblast: syncytiotrophoblast and cytotrophoblast. Microvillus membrane interacts with maternal blood and basal plasma membrane is in close proximity to basal lamina which separates the trophoblast from the vascular stromal core: fetal capillary(ies), Hofbauer cell(s), and fibroblast(s).
Figure 5: Transmission electron micrograph of sections through the cell surface of the syncytiotrophoblast. The section has been stained with ruthenium red (A) and Alcian blue (B). Arrows indicate caveolae in (A) and a caveole and a coated vesicle in (B). (From reference (Ockleford and Whyte 1977))
DIABETES

IPG P-type unchanged

Glucose - increased

G6P - increased

glycogen synthase

PRE-ECLAMPSIA

IPG P-type increased

Glucose - unchanged

G6P - ?

glycogen synthase phosphatase

glycogen synthase [active form]

glycogen synthase-P

ATP[kinase]

GLYCOGEN

increased in pre-eclampsia and diabetes

Figure 6: Factors regulating placental glycogen metabolism in pre-eclampsia and diabetes (from reference (Kunjara et al 2000a))
Chapter 1

IPGs in Placenta
INTRODUCTION

Introduction
An unknown placental factor, ‘factor X’ is postulated to be responsible for the pre-eclamptic disorder. It was hypothesised that this factor would be released by the placenta and enter the maternal circulation to cause maternal endothelial dysfunction (Redman 1993). Our group proposed a subfamily of IPGs as a candidate for this factor. This proposal is based on reasons explained in the General Introduction. IPGs are a family of second messengers mediating some of the actions of a wide range of factors and hormones including insulin. Although the precise chemical structures of the IPGs has not yet been determined, IPGs are identified as substances with molecular weights of 1000 to 2000 Da, consisting of phosphoinositol glycosidically linked to a glucosamine or galactosamine and containing several additional sugar residues (Saltiel and Cuatrecasas 1986, Saltiel et al 1986, Mato et al 1987a, Mato et al 1987b, Larner et al 1988, Merida et al 1988). This chapter concerns the presence of IPGs in chorionic villi of normal and pre-eclamptic human term placentae.

IPGs discovery
IPGs were in fact extracted as early as 1974 since Larner et al reported the production of a heat and acid extractable compound, released from skeletal muscle after insulin treatment, which inhibited cAMP-dependent protein kinase (Larner et al 1974).

In 1979, after additional purification steps, consisting of removal of nucleotides (adenosine triphosphate, diphosphate and monophosphate) by gel filtration on Sephadex G-25 columns, the fraction containing this putative insulin mediator was also shown to stimulate pyruvate dehydrogenase (PDH) activity and glycogen synthase phosphatase (Jarett and Seals 1979, Larner et al 1979a). The molecular size of the substance thought to be a peptide or derivatised peptide was estimated to be between 1000 to 1500 Da (Larner et al 1979a). (It was thought to be a peptide-like compound because it was free of nucleotide, exhibited absorbance at 230 nm and was ionic in character (Cheng and Larner 1985)). In 1981, Kiechle et al (Kiechle et al 1981) reported also the production of a low-molecular-weight factor from adipocyte plasma membranes in response to insulin that stimulated mitochondrial PDH.
After obtaining the insulin mediator from rabbit skeletal muscle (Jarett and Seals 1979, Larner et al 1979a) and rat adipocytes (Kiechle et al 1980, Popp et al 1980, Seals and Czech 1980, Seals and Jarett 1980, Kiechle et al 1981, Seals and Czech 1981), Saltiel et al purified, by using HPLC and Sephadex G25 columns, the insulin mediator from rat liver (Saltiel et al 1981). Then, the same group separated, by differential ethanol fractionation, two insulin mediators (Saltiel et al 1982). One stimulated PDH, the other inhibited adenylate cyclase. Both factors were released from isolated liver plasma membranes by physiological concentrations of insulin.

Soon after, Larner’s group also separated two distinct mediator activities (Thompson et al 1984, Cheng and Larner 1985). One inhibiting cAMP-dependent protein kinase (Thompson et al 1984) which also inhibited adenylate cyclase (Malchoff et al 1987) and the other which stimulated pyruvated dehydrogenase. The existence of multiple insulin mediators was therefore proposed (Saltiel et al 1982, Cheng and Larner 1985).

Following these findings, several groups confirmed the existence of these insulin mediators. New studies were initiated to elucidate their physical and chemical characteristics as well as on understanding the mechanism of their release. It was also shown that these compounds not only mediate the action of insulin but also other factors.

Further studies: chemical characteristics, release by PI-PLC, a glycolipid precursor
Saltiel et al (Saltiel and Cuatrecasas 1986) isolated two substances with an apparent molecular weight of approximately 1400 Da which modulated the activity of cAMP phosphodiesterase from hepatic plasma membranes. They were purified from liver plasma membrane after insulin stimulation. These substances were also generated by the addition of PI-PLC from Staphylococcus aureus to non-insulin stimulated membranes. They had similar properties and activities and could be resolved by ion-exchange chromatography and high voltage electrophoresis. They exhibited a net negative charge, even at low pH (1.9) (because of their behaviour in ion-exchange columns and migration on high voltage electrophoresis) which may be due to phosphate or sulphate groups. They were shown to be relatively polar on the basis of water solubility and inability to be retained on a reverse-phase chromatography resin. Limited oxidation by periodate indicated the presence of carbohydrate in the active
substance. This was also supported by the loss of activity after nitrous acid deamination, which implied the presence of glycosidically linked glucosamine in the active compound. The fact that PI-PLC treatment generated active products suggested that the released products contained inositol phosphate. The two substances could both be generated by PI-PLC hydrolysis of intact membranes and of an isolated putative glycolipid precursor. The glycolipid was chemically unidentified in this study but the author mentioned, in their next publication (Saltiel et al 1986), that it seemed related to carbohydrates-phosphate substances containing inositol and glucosamine.

Thus, the same group confirmed in the same year (Saltiel et al 1986) that these substances contain inositol and glucosamine with an approximate 1:1 stoichiometry. In this study $[^3]$H]inositol and $[^3]$H]glucosamine were incorporated in a murine myocyte cell line. The radioactivity was incorporated into a glycolipid with an Rf on TLC identical to that of previously described precursor. The radioactivity was decreased in the glycolipid after insulin or PI-PLC treatment and incorporated into fractions containing phosphodiesterase modulators.

Mato et al (Mato et al 1987a) generated from H35 hepatoma cells a compound of 800 to 3500 Da after PI-PLC treatment of a glycolipid radiolabelled with $[^3]$H]glucosamine. Nitrous acid deamination demonstrated that glucosamine was covalently linked through a phosphodiester bond to the glyceryl moiety of the purified glycolipid. The turnover of the non-N-acetylated glucosamine containing glycolipid was stimulated by insulin, which was proposed by these authors to stimulate a PLC acting on this glycolipid to 'generate intracellular mediators that seem to be responsible for regulating the activity of various key enzymes in insulin-sensitive metabolic pathways'.

It was also mentioned that the polar head group of this glycosphospholipid mimicked the effect of insulin on isoproterenol-stimulated phospholipid methyltransferase (Kelly et al 1986) and lipolysis (Kelly et al 1987) in rat adipocytes.

Further studies by this group gave more chemical information. They showed that this glycolipid, which is extracted from H35 cells and also rat liver membranes, contains chiro-inositol, galactose and three phosphate groups (Mato et al 1987b). Three different forms of the polar head group of the glycolipid of rat liver membranes were also reported (Merida et al 1988).
Lerner *et al* (Lerner *et al* 1988) purified insulin mediators, which stimulate PDH phosphatase, from rat liver. They contained mannose, galactosamine, and D-chiro-inositol. By using paper chromatography and gas chromatography/mass spectroscopy, it was found that nitrous acid deamination resulted in release of inositol phosphate, indicating that the galactosamine and D-chiro-inositol were linked. The presence of galactosamine rather than glucosamine was a novel finding.

*Two subfamilies: IPG A- and P type*

The large panel of effects (reviewed in (Jones and Varela-Nieto 1998, Varela-Nieto *et al* 1996) and Table 1.1) and chemical variations of these compounds have led to a general acceptance that IPGs constitute a family of compounds rather than a single compound with a definite chemical composition.

The group of mediators containing inositol, phosphate and a glycan moiety form a family of compounds which differ in chemical composition and biological effects. Two main structural groups of IPGs have been proposed: IPG type A type and IPG P-type (Rademacher *et al* 1994, Kunjara *et al* 1995). IPG A-type is composed of myo-inositol, hexose, glucosamine and phosphate; IPG P-type is composed of chiro-inositol, hexose, galactosamine and phosphate. The presence of Zn$^{2+}$ and Mn$^{2+}$ was also detected in IPG A- and IPG P-type respectively (unpublished results from our laboratory). They differ in their chemical composition as well as in their biological activities.

IPG A-type regulates the level of cAMP in cells by inhibiting adenylate cyclase and stimulating cAMP phosphodiesterase. It stimulates acetylCoA carboxylase and inhibits cAMP-dependent protein kinase A. IPG A-type also stimulates the proliferation of the cochleovestibular ganglion of the developing inner ear of the chicken (*Caro et al* 1997), mimicking the effect of IGF-I (*Leon et al* 1995). Additionally, IPG A-type is able to repress cAMP-mediated gene expression in rat hepatocytes (*Alvarez et al* 1991a, *Caro et al* 1997) and inhibits the release of leptin from rat adipocytes.

IPG P-type stimulates PDH phosphatase and glycogen synthase phosphatase. Its overall metabolic effect is to stimulate glycogen biosynthesis, contrasting with IPG A-type which stimulates lipogenesis. IPG P-type stimulates also the activation of the glycerol-3-phosphate acyltransferase (*Farese et al* 1994). Furthermore both type A
and P mediators are mitogenic when added to fibroblasts transfected with EGF receptors (Caro et al 1997). IPGs isolated by Mato and co-workers in 1987 (Mato et al 1987b, Mato et al 1987a) are attributed as IPG A-type based on effect criteria. Those isolated by Larner et al in 1988 (Larner et al 1988) belongs to the IPG P-type sub-family.

The purification procedure which separates IPG A-type from IPG P-type was first described by Larner's group (Nestler et al 1991) and was then used by our group with some modifications (Caro et al 1997, Kunjara et al 1999, Kunjara et al 2000a, Kunjara et al 2000b). This procedure is based on heat denaturation of protein, charcoal adsorption of nucleotides and anion exchange resin which yields both fractions separately. The fraction eluting from a column of AG1X8 anion-exchange resin with HCl at pH 2 contains chiro-inositol, galactosamine and phosphate (ie IPG P-type) (Fonteles et al 1996), the fraction eluting with HCl at pH 1.3 contains myo-inositol, glucosamine, galactose (ie IPG A-type) (unpublished results from our group).

In our laboratory, IPG A-type was found to antagonise IPG P-type in the stimulation of the PDH phosphatase (Nestler et al 1991, Kunjara et al 1999). This is proposed to be due to the metal content of IPGs. IPG A-type contains predominantly zinc whereas IPG P-type contains predominantly manganese.

**IPGs mimic insulin and other factors**

IPGs are mediators of insulin but also have non-insulin effects. The insulin effects are reviewed in (Varela-Nieto et al 1996, Jones and Varela-Nieto 1998) and shown in Table 1.1.

As shown, the insulin-mimetic biological effects of IPGs range from the positive and negative modulation of general cell metabolism (including lipolysis, lipogenesis, glycolytic flux, protein synthesis and cell proliferation) through to regulation of specific enzymes (phosphatases, phosphodiesterases, transferases and kinases).

Diverse IPG non-insulin effects have been reported. In contrast with insulin, IPG A-type inhibits the release of leptin in rat adipocyte cells. IPGs are also an important component of T-cell growth responses; they increase proliferation of mouse T cells induced by IL2 (Merida et al 1990). A cell proliferative effect is also shown in the case of erythropoeitin in rat erythroid progenitor cells, in which IPGs are able to
potentiate colony formation in the presence of suboptimal erythropoietin concentrations (Devery et al 1994). IPGs are also thought to have a role in the development and maintenance of the nervous system by mediating the effects of neurotrophic factors. Nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3, three neurotrophic factors sharing the same low affinity receptor, seem to modulate cell proliferation in the cochleovestibular ganglion through generation of IPGs after GPI hydrolysis (Represa et al 1991, Varela-Nieto et al 1991, Represa et al 1993). However, a non-IPG signal transduction pathway seems to be used when these factors have a differentiation effect (Varela-Nieto et al 1996).

Another example of a non-insulin effect of IPG is the positive modulation of *fos* and *jun* gene expression induced by IGF-I (Leon et al 1995). Table 1.2, from reference (Varela-Nieto et al 1996) indicates the growth factors and hormones other than insulin which stimulate GPI hydrolysis, and therefore are presumed to use the IPG/GPI signal transduction pathway.

The growth factors or hormones which use IPGs as mediators or which have been shown to act through GPI hydrolysis have their membrane receptors belonging to different categories. These include seven transmembrane domain receptors and tyrosine kinase receptors.

**IPG in signal transduction**

IPG/GPI signalling represents a novel signalling system which is different to the well-established signal transduction pathways involving generation of cyclic nucleotides, polyphosphoinositide hydrolysis and ion fluxes across the plasma membrane. However the precise mechanism of IPG generation and its exact downstream targets remain to be defined.

IPGs are defined as insulin second messengers because, as mentioned before, IPG release occurs after GPI hydrolysis upon insulin stimulation and they mimic some of the effects of insulin. The proposed principle mechanism of release of IPGs is presented on Figure 1.1. Following the receptor ligation of insulin, a phospholipase is activated, this later hydrolyses a glycolipid molecule, glycosylphosphatidylinositol (GPI), resulting in release of IPGs into the extracellular medium.
IPGs were first thought to be released inside the cell. 'An inward orientation of the insulin-target GPI was proposed. However, the only reason for this suggestion was the prejudice that an insulin mediator should be released directly into the cytoplasm of the cell, by analogy with other second messengers such as cyclic AMP or the inositol phosphates.' (From reference (Varela-Nieto et al 1996). Varela-Nieto et al (Varela-Nieto et al 1996) listed the 'Evidence favouring the hypothesis that IPG is generated extracellularly'. They include: '(1) its ability to mimic many of the actions of insulin when added exogeneously to intact cells; (2) its presence in the culture medium of several cell types after insulin treatment; (3) the fact that anti-IPG antibodies block the effects of insulin, IGF-I, EGF and the NGF family of neurotrophins in intact cells and (4) data obtained by using chemical labelling with $[^{14}C]$isethyonyl acetimidate indicate that the majority of IPG precursor, the insulin target GPI, is found at the outer surface at the cell in rat adipocytes and hepatocytes.'

The involvement of receptor tyrosine kinase in GPI hydrolysis has been proposed and is supported by both direct and indirect evidence. Firstly, insulin receptor tyrosine kinase domain-deficient cells prevented insulin-stimulated GPI hydrolysis (Suzuki et al 1992). Secondly, tyrosine kinase inhibitors are able to severely inhibit EGF-stimulated GPI hydrolysis (Clemente et al 1995). Additionally, complete functional receptors are required for TGFβ-mediated cell proliferation and growth inhibition as mutant receptors were not able to signal through the generation of IPG (Bogdanowicz et al 1996). Furthermore, in a knockout experiment where the synthesis of GPI was eliminated, insulin-stimulated glycogen synthesis in K562 cells was inhibited (Lazar et al 1994). In contrast, other studies suggest that the tyrosine kinase activity of the insulin receptor may not be required for the release of IPGs (Forsayeth et al 1987, Hawley et al 1989, Gottschalk 1991).

The mechanism by which IPGs enter the cell remains unknown. An IPG receptor or an uptake system at the plasma membrane has been postulated (Alvarez et al 1991b). Specific $[^{3}H]$galactose IPG uptake in isolated rat hepatocytes was studied. The uptake was temperature (37°C), concentration and time-dependent, and metabolic poisoning with KCN disrupted it. These observations imply that an active transport
system exists and suggests that a free diffusion of IPG through the plasma membrane does not occur.

As mentioned before, IPGs seem to mediate the action of a large panel of factors (see Table 1.1). However, it is unlikely that IPGs alone could achieve the full range of actions of this panel of factors, or even of just one factor. For illustration, as mentioned before, neurotrophic factors use the IPG/GPI signalling pathway when they have a proliferative effect, but switch to another pathway when they stimulate differentiation. On the other hand, because IPG effects are so numerous, Varela-Nieto et al (Varela-Nieto et al 1996) proposed that the expected multiplicity of free GPI and IPG types might explain the specificity of the response. 'If the different agonists stimulate the hydrolysis of distinct GPI types, the generation of signal specific IPG would induce differentiated cellular responses. Furthermore, another level of regulation could be accomplished if there are GPI types specific to tissue or if the different IPG types have distinct cellular targets'.

To date, the downstream targets of IPG messengers are not well defined. IPGs caused enhanced phosphorylation of some proteins and reduced phosphorylation of others (Alemany et al 1987). IPGs seem to act directly on intracellular enzymes such as specific serine/threonine protein phosphatase (Misek and Saltiel 1994), PDH phosphatase and cAMP-dependent protein kinase A. Recently, IPGs were suggested to be transporters of trace metal ion cofactors due to their effect on PDH phosphatase, their metal content and the effects of metal on activity of this enzyme (Kunjara et al 1999).

Generation of IPG
The release of IPG from GPI is due to the activation of a phospholipase which generates diglyceride in addition to IPG. It was initially suggested that this phospholipase was a phospholipase C (Varela-Nieto et al 1996, Field 1997, Stralfors 1997) since bacterial PI-PLC was used to generate IPGs (Saltiel et al 1986, Saltiel and Cuatrecasas 1986, Mato et al 1987a).

Specific GPI-PLC have been described in the African protozoan parasite Trypanosoma brucei (Mensa-Wilmot et al 1990, Armah and Mensa-Wilmot 2000) but not in mammals. GPI-specific PLD, an enzyme identified in mammals, has also
been proposed to be involved in the generation of IPG from GPI. Jones et al (Jones et al 1997) showed that free GPI was a substrate for GPI-PLD and the resulting IPG showed similarity to IPG produced by PI-PLC. Chapter 3 of this thesis examines GPI-PLD expression in placenta. The ability of the GPI-PLD enzyme to generate IPG from free-GPI is studied in Chapter 4.

**IPG cellular location**

IPGs are released from GPI present in the plasma membrane. As mentioned before, the orientation of GPI in the plasma membrane was originally thought to be intracellular, thus generating IPGs directly in the cytoplasm (Saltiel et al 1986). It was then accepted that the IPG precursors were located in the outer leaflet of the plasma membrane (Alvarez et al 1988, Varela et al 1990). Evidence of extracellular release of IPGs and their subsequent biological effects inside the cell led to a hypothesis that IPGs are transported into the cell by an unknown transporter which presently has not been identified (Varela et al 1990, Alvarez et al 1991b).

More precisely, it has been reported that in rat adipocytes the glycolipid precursor of IPGs seems to be localised to a specific part of the plasma membrane, the caveolae (Parpal et al 1995).

To date, nothing in the literature has examined the precise location of IPGs inside the cell.

**IPGs in placenta: presence and function**

The IPG signalling system is widely distributed throughout many organs. Both IPG A-type and IPG P-type were found in rat liver, muscle, adipose tissue, heart, kidney, brain (Kunjara et al 1995), human liver (Caro et al 1997) and human placenta (Suzuki et al 1984, Kunjara et al 2000a). A tissue-specific distribution of IPG generation has been proposed (Kunjara et al 1995). In addition, IPG activity was generated from murine circulating T cells (Gaulton et al 1988), human mononuclear cells from peripheral blood (Suzuki et al 1984), and murine erythrocytes (Caro et al 1996). IPGs were also extracted from human urine (Kunjara et al 2000a).

From human placenta, both IPG A- and P type were extracted (Kunjara et al 2000a). IPG P-type activity was higher when IPGs were extracted from pre-eclamptic placenta. A role of placental IPG P-type is proposed in the regulation of glycogen.
synthesis in normal and pre-eclamptic placenta (discussed in more detailed in General Introduction).

Placental IPG activity was first reported by Suzuki et al (Suzuki et al 1984). They extracted a 1500 Da insulin mediator, which activated pyruvate dehydrogenase and suppressed glucose-6-phosphatase activity, from a preparation of plasma membrane. The method of membrane preparation used implies that the membrane fraction contains not only the syncytial brush border membrane but also the syncytial basal plasma membrane as well as plasma membrane of non trophoblastic cells present in the placenta.

In addition, an effect of IPGs was reported in human placenta. Both IPG A- and P type, extracted from bovine liver, modified human placental steroidogenesis. They suppressed aromatase activity and stimulated 3β-hydroxysteroid dehydrogenase in cultured cytotrophoblast cells (Nestler et al 1991)

**IPGs and pathological conditions**

As stated in the General Introduction, IPG P-type activity was found to be higher in the placenta and in the urine of pre-eclamptic patients compared to normal pregnant controls. Defects of IPG release or GPI hydrolysis have been reported in pathological conditions other than pre-eclampsia such as diabetes and obesity, and in conditions with altered metabolism such as aging.

Hepatocytes from obese and aged rats were shown to display altered GPI metabolism. In both cases the amount of GPI present and its extent of hydrolysis in response to insulin is reduced. In addition, the uptake of IPG and insulin-stimulated glycogen synthesis was found to be inhibited (Sanchez-Arias et al 1993, Sanchez-Gutierrez et al 1994).

In metabolically-altered rat adipocyte cells from two different animal model of diabetes (the type II diabetic Goto-Kakizaki rats and the streptozotocin-diabetic rats), insulin-stimulated GPI hydrolysis has also been shown to be impaired (Macaulay and Larkins 1990, Farese et al 1994). In the type II diabetic Goto-Kakizaki rats model (Farese et al 1994), the IPG sub-type has been defined. The defect affected the release of the chiro-inositol containing IPGs (the IPG P-type).
Similarly, in type II diabetic humans, a lower level of IPG P-type compared to IPG A-type was reported in urine (Kennington et al 1990). And more recently, our group observed that the IPG A : IPG P ratio is raised in non-insulin-dependent diabetes mellitus (NIDDM) (Kunjara et al 1999).

In addition, a role of IPGs is proposed in the onset of the obesity. The fact that IPG A-type decreased the release of the satiety hormone leptin from adipocyte cells (Kunjara et al 2000b) led the authors to suggest that this effect could be part of an early initiation phase in the eventual development of obesity.

 Reasons for investigating IPGs in the normal and pre-eclamptic placenta

IPGs have been extracted from placenta but no information concerning their location in the heterogeneous organ is available. No one has ever looked at IPG in microvilli. To complete and support the hypothesis of IPGs as a possible placental factor X responsible for the pre-eclamptic disorder, IPGs could be attached to microvilli and may cause the disorder circulating in the blood associated with the shed microvilli. The detection of IPGs in microvilli constitutes a first step in the understanding of the mechanism of release of IPGs in order to explain abnormal IPG P activity in pre-eclampsia.

IPGs were recently found to modulate leptin release from rat adipocytes (Kunjara et al 2000b). As leptin is not only made by adipocyte tissue but also placenta (Masuzaki et al 1997) and both IPG P activity (Kunjara et al 2000a) and leptin levels (Mise et al 1998) are high in pre-eclampsia, it was of interest to know whether a similar control of the release of leptin occurs in placenta.

Aim of the chapter

Therefore, the work presented in this chapter concerns the location of IPGs in chorionic villi of normal and pre-eclamptic placenta, the investigation of IPG presence in microvilli, and the study of a possible IPG effect on placental leptin release.
Figure 1.1: Simple model of IPG release. After insulin stimulation, GPI-PLD cleaves GPI. IPGs are thus released in the extra-cellular medium and then enter the cell where they are active.
Table 1.1: Insulin-like effects of IPGs (from Varela-Nieto et al 1996)

<table>
<thead>
<tr>
<th>Intact Cells</th>
<th>Biological activities</th>
<th>Effects</th>
<th>Biological sources of IPGs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipolysis</td>
<td>Inhibition</td>
<td>Rat liver; Calf haemodialysate; T. brucei; Plasmodium</td>
</tr>
<tr>
<td></td>
<td>Lipogenesis</td>
<td>Stimulation</td>
<td>Rat liver; Rat testes; Calf haemodialysate; Rat adipocytes; Plasmodium</td>
</tr>
<tr>
<td></td>
<td>Phospholipid methyltransferase</td>
<td>Inhibition</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Steroidogenesis</td>
<td>Stimulation</td>
<td>Bovine liver</td>
</tr>
<tr>
<td></td>
<td>Glucose transport</td>
<td>Stimulation</td>
<td>Calf haemodialysate; Rat fibroblasts; Rat adipocytes</td>
</tr>
<tr>
<td></td>
<td>Acetyl-CoA carboxylase activity</td>
<td>Stimulation</td>
<td>Rat hepatoma cells</td>
</tr>
<tr>
<td></td>
<td>Glycogen phosphorylase a activity</td>
<td>Inhibition</td>
<td>Rat liver; Human erythrocytes</td>
</tr>
<tr>
<td></td>
<td>Pyruvate kinase activity</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Glucose oxidation</td>
<td>Stimulation</td>
<td>Rat liver; Human T cells; Plasmodium</td>
</tr>
<tr>
<td></td>
<td>Glucose production</td>
<td>Inhibition</td>
<td>T. brucei</td>
</tr>
<tr>
<td></td>
<td>Lactate accumulation</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Glycogen synthetis</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Tyrosine aminotransferase activity</td>
<td>No effect</td>
<td>Rat hepatoma cells</td>
</tr>
<tr>
<td></td>
<td>Protein phosphorylation</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>cAMP levels</td>
<td>Inhibition</td>
<td>Rat liver; T. brucei</td>
</tr>
<tr>
<td></td>
<td>Fructose-2,6-2-P levels</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Ion channels</td>
<td>Modulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Amino acid transport</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Protein synthesis</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Specific mRNA levels</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>DNA and RNA synthesis</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Cellular proliferation</td>
<td>Stimulation</td>
<td>Rat hepatoma cells; Rat liver</td>
</tr>
<tr>
<td></td>
<td>Insulin secretion</td>
<td>Inhibition</td>
<td>Rat liver</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cellular extracts</th>
<th>Enzymatic activity or phosphorylation</th>
<th>Effects</th>
<th>Biological sources of IPGs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP phosphodiesterase</td>
<td>Stimulation</td>
<td>Rat adipocytes; Murine myocytes; Rat liver; Bovine liver</td>
</tr>
<tr>
<td></td>
<td>Pyruvate dehydrogenase (PDH)</td>
<td>Stimulation</td>
<td>Rat adipocytes</td>
</tr>
<tr>
<td></td>
<td>PDH phosphatase</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Glucose-6-phosphatase</td>
<td>Inhibition</td>
<td>Rat liver; T. brucei</td>
</tr>
<tr>
<td></td>
<td>Fructose-1,6-bisphosphatase</td>
<td>Inhibition</td>
<td>T. brucei; Porcine kidney</td>
</tr>
<tr>
<td></td>
<td>Adenylate cyclase</td>
<td>Inhibition</td>
<td>Bovine liver; Rat liver</td>
</tr>
<tr>
<td></td>
<td>cAMP kinase</td>
<td>Inhibition</td>
<td>Rat liver; Chicken embryo, Mouse T cells</td>
</tr>
<tr>
<td></td>
<td>Casein kinase II</td>
<td>Biphase</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Glycerol-3P acytransferase</td>
<td>Stimulation</td>
<td>Bovine liver</td>
</tr>
<tr>
<td></td>
<td>ATP citrate lyase</td>
<td>Stimulation</td>
<td>Rat liver</td>
</tr>
<tr>
<td></td>
<td>Galactolipid sulfotransferase</td>
<td>Inhibition</td>
<td>Rat testes</td>
</tr>
</tbody>
</table>
Table 1.2: Regulation of GPI hydrolysis (from Varela-Nieto et al 1996)

<table>
<thead>
<tr>
<th>Signal</th>
<th>Effect</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>Stimulation</td>
<td>BC$_3$H-1 myocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H35 hepatoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T-lymphocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat hepatocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat adipocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CHO cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human fibroblasts</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Stimulation</td>
<td>BC$_3$H-1 myocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Otic vesicle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>EGF</td>
<td>Stimulation</td>
<td>BC$_3$H-1 myocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibroblasts (EGFR T17)</td>
</tr>
<tr>
<td>NGF</td>
<td>Stimulation</td>
<td>PC-12 cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CVG</td>
</tr>
<tr>
<td>BDNF &amp; NT-3</td>
<td>Stimulation</td>
<td>CVG</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Stimulation</td>
<td>Rabbit articular chondrocytes</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Stimulation</td>
<td>Rat granulosa cells</td>
</tr>
<tr>
<td>FSH</td>
<td>Stimulation</td>
<td>Rat granulosa cells</td>
</tr>
<tr>
<td>Chorionic gonadotropin</td>
<td>Stimulation</td>
<td>Rat granulosa cells</td>
</tr>
<tr>
<td>Epo</td>
<td>Stimulation</td>
<td>Rat erythroid progenitor cells</td>
</tr>
<tr>
<td>ACTH</td>
<td>Stimulation</td>
<td>Adenal glomerulosa cells</td>
</tr>
<tr>
<td>TRH</td>
<td>Stimulation</td>
<td>Pituitary lactotrophes</td>
</tr>
<tr>
<td>TSH</td>
<td>Stimulation</td>
<td>Pig thyroid cells</td>
</tr>
<tr>
<td>IL-2</td>
<td>Stimulation</td>
<td>T and B-lymphocytes</td>
</tr>
<tr>
<td>IL-4</td>
<td>Blockade</td>
<td>B-lymphocytes</td>
</tr>
</tbody>
</table>

Abbreviations: ACTH, adrenocorticotropic hormone; BDNF, brain-derived neurotrophic factor; EGF, epidermal growth factor; Epo, erythropoietin; FSH, follicle-stimulating hormone; IGF-I, insulin-like growth factor I; IL, interleukin; NT-3, neurotrophin-3; TGF-β1, transforming growth factor-β1; TRH, thyrotropin releasing hormone; TSH, thyrotropin.
MATERIALS & METHODS

1- Immunohistochemistry

Materials
The placentae used for immunohistochemistry were from Chelsea and Westminster Hospital. Villi tissue were prepared from 3 normal (38 ± 2 SD weeks) and 3 pre-eclamptic (36 ± 2 SD weeks) term placentae. Pre-eclampsia was diagnosed by clinicians of the hospital, as defined according to the criteria of the national high blood pressure education program working group (National High Blood Pressure Education Program 1990). Women were proteinuric (> 300 mg / 24 hours) and had new hypertension at or above ≥ 140/90 mmHg. Placentae were collected and embedded by Dr S Sooranna, Imperial College School of Medicine. 2D1 Monoclonal IgM anti-IPG was a gift from Prof J Mato, University of Navarra. Goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) and purified mouse IgM were from Dako Corp. (Slough, England). Optimum Cutting Temperature (OCT) medium and haematoxylin were from Raymond A. Lamb (London, England). Phosphate buffer saline (PBS) tablets, 3,3′-diaminobenzidine tetrahydrochloride and normal goat serum were from Sigma Aldrich Ltd (Poole, England). Styrolyte mounting medium and iso-pentane was from BDH Ltd (Poole, England). Immersion oil was from Emmersonol (Leitz, Germany). Films were Kodak Ektachrome Professional 400 ASA films.

Methods
The detection and localisation of IPGs in human placental villi was determined by immunohistochemistry using 2D1, an anti rat IPG monoclonal IgM antibody. 2D1 was raised in mouse against rat liver GPI previously treated with GPI-PLC. It was screened with liver IPG A-type and also recognises placental IPG P-type. Pieces of fresh placental villi from normal and pre-eclamptic human term placentae were embedded in OCT medium onto a cork, frozen in super-cooled isopentane and stored at -80°C. Serial cryostat sections (6 μm) were cut using a Bright Instrument (Company Ltd Huntingdon, England) and stored at -20°C. Prior to labelling with antibodies, the sections were allowed to equilibrate at room temperature, and non-
specific absorption was blocked with 20% normal goat serum (NGS) in PBS for 60 min at room temperature. Immunostaining was via indirect immunohistochemistry using the mouse monoclonal IgM antibody diluted to 10 μg/ml in PBS containing 2% NGS. The sections were incubated for 60 min at room temperature in a humidified atmosphere followed by three brief washes of PBS. Controls for the antibody were conducted using a purified mouse IgM at the same concentration under the same conditions. The secondary antibody detects the presence of the primary antibody and hence the original antigen of interest, the IPGs. The secondary antibody was a goat anti-mouse IgM conjugated to horseradish peroxidase (HRP) used at a dilution of 1/100. It was placed onto the tissue sections and incubated for another 60 min at room temperature in a humidified atmosphere and washed. The HRP sections were then incubated for 10 min with 3,3’-diaminobenzidine tetrahydrochloride solution to develop the brown dye corresponding to the presence of HRP; they were stained with Haematoxylin and mounted in Styrolyte mounting medium. Specimens were then examined through immersion oil and photographs were taken.

2- Syncytial microvillous membrane preparations
Preparation of the microvillous membrane of the syncytiotrophoblast

Materials
Normal and pre-eclamptic placentae used to prepare microvilli, and to extract GPI (see Chapter 2), were obtained from UCL Hospital. As before, pre-eclampsia was diagnosed by the clinicians of the hospital, as defined according to the criteria of the national high blood pressure education program working group (National High Blood Pressure Education Program 1990). Women were proteinuric (> 300 mg / 24 hours) and had new hypertension at or above ≥ 140/90 mmHg. Placentae were collected immediately after delivery. PBS tablets were obtained from Sigma Aldrich Ltd (Poole, England).
**Methods**

Microvilli were simultaneously prepared following modified methods of Eaton and Oakey (Eaton and Oakey 1994) and Rebourcet et al (Rebourcet et al 1998) based originally on a method described by Smith et al (Smith et al 1974). The villous tissue was removed from the chorionic and basal plates with scissors. Chunks of tissue were placed in ice cold PBS to remove much of the blood from the intervillous space. The tissue was then blotted and its wet weight was determined. Subsequent procedures were performed at 4°C.

About 20 g of tissue was then washed with PBS and carefully transferred to a beaker containing 150 ml of PBS (pH 7.4). The mixture was gently stirred with a magnetic stirrer for 1 h. Large cell debris was removed by filtering through a nylon sieve, and the filtrate was centrifuged at 800 g for 15 min. The resulting supernatant was centrifuged at 10 000 g for 10 min to remove larger particles and intracellular debris. The microvilli were collected by centrifugation of the supernatant at 100 000 g for 1 h.

The pellet obtained was resuspended in a minimal volume of 10 mM Tris HCl (pH 7.4) using a Dounce homogeniser and stored at -80°C until required.

The protein content of the microvillous membrane preparations was determined for quantification. Alkaline phosphatase activity was also determined to check the enrichment in microvilli of the preparation compared to an homogenate of placenta, as alkaline phosphatase is a placental microvilli marker (Booth et al 1980).

**Protein assay**

**Materials**

Folin & Ciocalteu’s phenol reagent was from BDH Ltd. (Poole, England). Bovine serum albumin was from Sigma Aldrich Ltd (Poole, England). All other reagents were of the highest purity available (Analytical grade).

**Method**

The protein concentrations of intact placenta and microvillous membrane preparations were measured by the method of Lowry et al (Lowry et al 1951) which was modified for use as a microassay.
Four solutions A, B, C and D were prepared. Solution A consists of 2% Na$_2$CO$_3$ in 0.1N NaOH, solution B is 1% CuSO$_4$ with 2% NaK tartrate, solution C is 50 ml of solution A and 1 ml of solution B and solution D is 10 ml of Folin and Ciocalteu’s phenol reagent diluted into 13.6 ml of ultra pure water.

40 µl of solution C and 10 µl of sample were incubated in a well of a 96-well plate for 10 min, 5 µl of solution D was then added. After 40 min, absorbance was read at 650 nm with a 96-well plate reader (Molecular Devices Thermomax microplate reader in conjunction with Softmax version 2.35)

Bovine serum albumin (1 mg/ml maximum concentration) was used for the standard curve.

Alkaline phosphatase assay

Materials

p-nitrophenyl phosphate (pNPP) was obtained from Sigma Aldrich Ltd (Poole, England).

Method

The alkaline phosphatase activity was measured by ELISA. In presence of alkaline phosphatase, pNPP produces a soluble end product that is yellow in color and can be read spectrophotometrically at 405 nm.

In a 96-well plate, 5 µl of membrane preparation or 5 µl of distilled water for the blank was incubated with 95 µl of p-nitrophenyl phosphate (pNPP) solution which consisted of 1 mg/ml pNPP in 0.1 M glycine buffer (pH 10.4) containing 1 mM MgCl$_2$ and 1mM ZnCl$_2$.

After 40 min incubation, the reaction was stopped by the addition of 25 µl of 3 N NaOH and the absorbance was read at 405 nm

In this chapter, syncytial microvillous membrane preparations were used to investigate the presence of IPGs in this part of the placenta. These membrane preparations were also used in the following chapters.
3- IPGs extraction procedure

Materials
AG1X8 resin was obtained from Bio-Rad Laboratories Ltd (Hertfordshire, UK). β-Mercaptoethanol and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Aldrich Ltd (Poole, England). Cellulose was obtained from BDH Ltd (Poole, England).

Methods
To determine if homogenates of placenta and microvilli preparations contained IPG-like activities, IPG A-type and IPG P-type were extracted as described previously for rat and bovine tissue (Larner et al 1989, Nestler et al 1991). In our laboratory this technique has previously been used to extract IPGs from human liver (Caro et al 1997), urine and placenta (Kunjara et al 2000a).

The homogenate of placenta was prepared by pulverising 1g of placenta using a mortar placed in liquid nitrogen. The powder obtained was homogenised in 15 ml of extracting buffer, consisting of 50 mM formic acid containing 1 mM EDTA and 1 mM β-mercaptoethanol. A volume of microvilli preparation whose protein content had been previously determined was diluted in 15 ml of the same extracting buffer (50 mM formic acid, 1 mM EDTA and 1 mM β-mercaptoethanol). Extractions were done with microvilli containing 0.4 mg and 1 mg of protein, the volume of starting microvilli preparations was never higher than 1ml.

Placental microvilli and homogenate were boiled in the extracting buffer for 3 min and then cooled on ice for 30 min. After centrifugation at 29,500 g for 90 min, the deproteinized clear supernatant was treated with charcoal 0.150g, stirred for 15 min on ice to absorb nucleotides, and centrifuged. The clear supernatant was diluted 10 fold with distilled water and the pH was adjusted to 6.0 with NH₄OH. This solution was mixed with AG1X8 resin (formate form, 24ml settled volume) and stirred overnight at 4°C.

After an overnight absorption, the AG1X8 resin was poured into a chromatography column and washed with distilled water.

The column was then washed with 55ml HCl (pH 3.0), and eluted with 100ml HCl 10mM (pH 2.0) obtaining IPG P-type, followed by 100 ml HCl 50mM (pH 1.3) obtaining IPG A-type. Each eluate was adjusted to pH 4.0 with NH₄OH and
evaporated to a small volume in a rotary evaporator, followed by lyophilization to dryness. The freeze-dried fractions were stored at -80°C until required.

Some of the IPG fractions were subjected to cellulose chromatography to further separate lipid or other contaminants. Cellulose powder was suspended in 50% ethanol/water and 1 ml of the suspension was poured into columns. The column of cellulose was washed with 5 ml water followed by 5 ml butanol/water/ethanol (B/W/E in the ratio of 4/1/1). IPGs were dissolved in 500 μl of the 4/1/1 mixture and loaded on the top of the column. The column was eluted successively with 5 ml B/W/E, followed by 5 ml methanol, 5 ml water, and 10 ml 50 mM HCl (pH 1.3). The eluates were concentrated, freeze-dried and tested.

The IPG activity, as assessed by the PDH phosphatase and cAMP-dependent protein kinase A assays, was found to elute in the acid fraction. This suggests that IPGs are retained by cellulose and there is no IPG like activity eluting in the organic solutions. The IPGs presented in the results were not subjected to further purification.

4- Measurement of IPG A-type and IPG P-type activity

The activity of IPG A type and IPG P-type in the extracts separated from intact placenta and placental microvilli preparations was measured using specific bioassay procedures. An assay measuring cell proliferation, an assay measuring the inhibition of cAMP dependent protein kinase A and an assay measuring the stimulation of PDH phosphatase were used. For the bioassay procedures, the samples were dissolved on the day of the use. They were suspended in 80 μl distilled water when tested in the PDH phosphatase assay and in the cAMP-dependent PKA assay, and in 80 μl HBSS when tested in the cell proliferation assay. The pH of the samples was adjusted to 7 with KOH in order to maintain cell viability.

a- Cell proliferation assay

Cell culture

Materials

EGFR T17 cells were a gift from Prof Mato, University of Navarra. Dulbecco’s modified Eagle medium (DMEM), penicillin/streptomycin solution, glutamine and
Hanks' balanced salt solution (HBSS) were from Gibco BRL - Life Technologies Ltd (Paisley, UK). Trypsin was from Sigma Aldrich Ltd (Poole, England).

**Method**
The cells used to assay the ability of IPG to activate fibroblast cell proliferation were EGFR T17 fibroblasts. They were NIH-3T3 fibroblasts transfected with the human epidermal growth factor receptor (Velu *et al* 1987).

Cells were routinely grown in flasks containing 20 ml of medium, which consisted of DMEM containing 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humid atmosphere of 5% CO₂.

When they reached 80% confluence, the fibroblasts were subcultured as follows:

Once the medium was removed from the flasks, the cells were washed twice using 10 ml of HBSS, then 5 ml of trypsin-EDTA solution was used to detached the cells during a 5 min incubation at 37°C. The trypsin reaction was stopped by adding serum which was contained in the medium. In order to remove trypsin, the suspension was centrifuged at 1400 rpm for 5 min and the cell pellet was resuspended in growth medium by gentle pipetting.

Five per cent of the cells of one flask were used to prepare a new culture by seeding into a new flask.

**Measurement of cellular proliferation in EGFR T17 fibroblasts**

**Material**

[^H]Thymidine was from Amersham Pharmacia Biotech (Uppsala, Sweden).

**Method**

To evaluate fibroblast cell proliferation, incorporation of[^H]thymidine was measured.

After trypsin treatment, an aliquot of cells was taken and counted in order that 10⁴ cells in 100 µl of growth medium were plated into each well of a 96-well microtiter plate. After 24 h the medium was removed, the cells were washed twice with at least 100 µl of HBSS and 100 µl of serum free medium was added. Twenty-four hours later, the medium was withdrawn and replaced with medium containing serum or IPG at different final concentrations. Control wells contained just medium without
serum or IPGs. Under these conditions, the cells were incubated for about 18-20 h, after which $[^{3}H]$thymidine (1 μCi/well) was added to each well for 4 h. At the end of this treatment, the medium was removed and the cells were trypsinized with 50 μl well for 10 min. The radioactivity incorporated in cellular DNA was then determined using a cell harvester and scintillation counter.

b- cAMP dependent protein kinase A assay

Materials

The colorimetric PKA assay kit and cAMP dependent protein kinase A were from Pierce (Rockford, USA). The kit contained a solution of $5 \times$ reaction buffer (10 mM ATP, 50 mM MgCl$_2$, 0.01% Triton X-100, and 100 mM Tris(hydroxymethyl)-amino methane, pH 7.4), a solution of $5 \times$ activator solution (500 mM cyclic AMP in water), a kemptide LRRASLG (which is a PKA substrate) labeled with a fluorescent probe, PKA dilution buffer (which is, when reconstituted with water and glycerol, 25 mM Tris, 0.15 M sodium chloride, pH 7.2, in 50 % glycerol), Spinzyme affinity separation units (units containing a proprietary affinity membrane specific for the binding of phosphorylated products), phosphopeptide binding buffer (0.1M sodium acetate, 0.5 M sodium chloride and 0.02% sodium azide, pH 5.0) and phosphopeptide elution buffer (0.1M ammonium bicarbonate, pH 8.0, containing 0.02% sodium azide).

Method

IPG inhibits cAMP-dependent protein kinase A. This colorimetric kit is used to measure this inhibition by incubating PKA with a labelled non-phosphorylated (substrate) in the presence or absence of IPG.

The peptide substrate (5 μl), labelled with a brightly colored fluorescent dye, was mixed with reaction buffer (5 μl) and activator solution (5 μl) and incubated for 30 min at 30°C with 5 μl of PKA containing 1U of the enzyme (1 unit will transfer 1 pmole of phosphate to dephosphorylated casein per minute per mg of kinase) with or without IPG sample (5 μl of 80 μl extracts or as mentioned). As a control the same reaction without addition of kinase was performed. Dilution buffer was added when necessary to ensure a total volume of 25 μl. After incubation, 20 μl of the reaction
mixture was applied to a separation unit containing the affinity membrane. The membrane binds specifically the phosphorylated substrate while unreacted (non-phosphorylated) substrate passes through the support when washed with binding buffer. Two washes of 250 μl were performed. After transferring the membrane-containing bucket to a new receptacle, the bound, phosphorylated substrate was eluted from the membrane twice with 250 μl of elution buffer. Quantitation of the phosphorylated product (following transfer to 96 well plates) was accomplished by measuring its absorbance at 565nm using a Molecular Devices Thermomax microplate reader in conjunction with Softmax version 2.35. The percentage of inhibition of PKA induced by IPGs is calculated on the basis that 1U PKA represents 100% activation and therefore 0% inhibition. In the absence of PKA, the inhibition is 100%.

c- Pyruvate dehydrogenase phosphatase activity assay

*Materials*

Pyruvate Dehydrogenase complex (PDC) and Pyruvate Dehydrogenase (PDH) Phosphatase were prepared from bovine heart by Dr Kunjara in the laboratory. ATP (disodium salt) and NAD were purchased from Roche Diagnostics Ltd (Lewes, East Sussex, UK). DTT, EGTA, BSA (Fatty Acid Free), NaF, TPP, Na pyruvate and Coenzyme A were obtained from Sigma Aldrich Ltd (Poole, Dorset, UK).

*Method*

A schematic representation of the PDH phosphatase assay. Active PDH is first inactivated by ATP. Inactive PDH is then converted into an active form by stimulation of PDH phosphatase induced by IPG P-type. Active PDH is able to convert pyruvate and NAD into Acetyl Co A and NADH which can be measured spectrophotometrically.
This assay is used to measure IPG P-type bioactivity upon the stimulation of PDH phosphatase. As illustrated in the Scheme above, IPG P-type activates PDH phosphatase which converts the inactive phosphorylated form of PDH into the active non-phosphorylated form. The active PDH then catalyses the conversion of pyruvate and NAD into Acetyl CoA and NADH. Thus the activation of PDH was determined spectrophotometrically by measuring the rate of production of the reduced form of NADH. Before stimulation by PDH phosphatase, PDH was inactivated by incubation with ATP.

The method used is described by Caro et al (Caro et al 1997). These measurements were done in collaboration with Dr Kunjara.

The pyruvate dehydrogenase complex (PDC), a preparation of active PDH (non-phosphorylated active form of pyruvate dehydrogenase) and the soluble PDH phosphatase, were isolated from bovine heart mitochondria. These enzymes were prepared by Dr Kunjara as described by Lilley et al (Lilley et al 1992). PDC and PDH phosphatase were stored in 50 µl and 100 µl aliquots respectively at -80°C until used. The initial activity of PDH was 20 units/ml (1 unit of enzyme produces 1µmol NADH/min). This value was reduced to less than 1% of the original value after inactivation with ATP. Inactivation was carried out by mixing 0.1 ml of PDC with 0.4 ml of PDC inactivation mixture containing 20 mM potassium phosphate buffer at pH 7.0, 1 mM ATP, 1 mM DTT, 0.1 mM EGTA, and 1 mM MgCl₂ and incubating at 30°C for 15 min. Once inactivated, the PDC was stored on ice and used for 10 assays.

A sample of inactivated PDC (50 µl) was preincubated at 30°C with 200 µl of PDH phosphatase assay mixture containing 1mg/ml fat-free bovine serum albumin, 10 mM MgCl₂, 0.1 mM CaCl₂, and 1mM dithiothreitol in 20 mM potassium phosphate buffer, pH 7.0, for 3 min. 10 µl of IPG solution and 10µl of the PDH phosphatase were then added and the incubation was continued for a further 3 min. The reaction was stopped by adding 135 µl of 300 mM sodium fluoride. The activated PDH activity was then determined spectrophotometrically by measuring the rate of production of NADH as follows. A volume of 200 µl of the stopped reaction was added to 1ml of reaction mixture containing 50 mM potassium phosphate buffer at pH 8.0, 2.5 mM of the oxidized form of NAD, 0.2 mM TPP, 0.13 mM coenzyme A,
0.32 mM DTT, and 2mM sodium pyruvate. The production of NADH was followed using absorbance at 340 nm for 7 min.

The activation of PDH induced by IPG P-type is presented as percentage activation above the basal level of activity in the absence of added IPG.

5- Measurement of the effect of IPGs on the release of leptin from BeWo cells
In order to know whether IPGs modulate the release of placental leptin, BeWo cells, a placental cell line, were incubated in the presence and absence of IPGs and leptin levels were determined in culture medium using a sensitive human leptin radioimmunoassay (RIA) kit.

a- BeWo cell culture

Materials
BeWo cells were a gift from Dr S Sooranna, Imperial College School of Medicine. RPMI medium was obtained from Sigma Aldrich Ltd (Poole, England). Calf serum and penicillin-streptomycin were obtained from Gibco BRL - Life Technologies Ltd (Paisley, UK).

Method
The culture of BeWo cells was performed by Dr JN Schofield, University College London. BeWo cells were grown in RPMI medium supplemented with antibiotic penicillin and streptomycin and 10 per cent calf serum under standard culture conditions (5% CO₂, humidified 37°C incubator).

b- Measurement of leptin secretion

Materials
Sensitive human leptin RIA kit was from Linco Research (S¹ Charles, USA). It included an assay buffer (0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08 % Sodium Azide, 0.05 % Triton X-100, and 1 % RIA grade BSA), a sensitive human leptin antibody produced in the guinea pig, ¹²⁵I-labelled human leptin (<1µCi/10ml), sensitive human leptin standards (0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10 ng/ml), quality controls and precipitating reagent.
Both IPG A-type and IPG P-type were prepared from microvillous membranes as described above (page 50). Insulin was obtained from Sigma Aldrich Ltd (Poole, England). Concentrator filtering columns were Vivaspin columns from Vivascience (Binbrook, England).

Method

Cells were plated at $1.2 \times 10^6$ cells/well in 12-well-plate and grown in an incubator overnight. They are then washed with PBS and incubated for 6 hours at 37°C in 2.5 ml of Krebs buffer (0.01 M Hepes, 0.2 M NaH$_2$PO$_4$, 0.01 M NaHCO$_3$, 0.13 M NaCl, 5 mM KCl, 1 mM MgSO$_4$ and 2.5 mM CaCl$_2$) containing 0.1% BSA and 10 mM D-glucose without (nil treatment) or with 4 units per ml of IPG A-type, 4 units per ml of IPG P-type or 10 nM insulin. (One unit of IPG is defined as the amount causing a 50 % inhibition of PKA). The 2.5 ml of medium was recovered and concentrated through filtering columns (10 000 MW) until obtaining a volume of 100 μl. Leptin content was measured in these samples and in filtered medium which was not incubated with cells (control). Each determination was in triplicate. The unknown concentration of leptin in samples is determined in the RIA assay by measuring how much of a known amount of the radioactively labelled hormone, $^{125}$I –human leptin, binds to a fixed quantity of anti-leptin antibody in the presence of leptin. This competition reaction is calibrated by constructing a standard curve (0.05 ng/ml to 10.0 ng/ml). The assay takes three days. The first day, 100μl of standards or samples are incubated with 100 μl of antibody in a 300 μl total volume, assay buffer (0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08 % Sodium Azide, 0.05 % Triton X-100, and 1 % RIA grade BSA). There were tubes without antibody to determine non-specific binding and tubes without cold leptin to determine total binding. Tubes were vortexed, covered and incubated overnight (20-24 hours) at room temperature. The next day, 100 μl of $^{125}$I-human leptin was added to all tubes. 100 μl in duplicate of this solution was kept to determine the total amount of radioactivity. Tubes were again vortexed, covered and incubated overnight (20-24 hours) at room temperature. The following day, 1.0 ml of cold (4°C) precipitating reagent was added. Tubes were vortexed, incubated 20 minutes at 4°C and centrifuged at 4°C for 20 minutes at 3,000 g. The supernate was then decanted and pellets counted in a gamma counter for 3 minutes. The concentration of human leptin
in unknown samples was calculated by determining the percentage of binding for each standard and sample. The results are calculated as leptin released per $1.2 \times 10^6$ cells. The difference was estimated using the paired t test, each mediator being compared with the nil treatment sample.

6- Measurement of the effect of IPGs on the proliferation of BeWo cells

It is established that IPGs stimulate the proliferation of EGFR T17 cells. Incorporation of thymidine into these cells is measured to detect IPG activity in this thesis. We used the same technique of measurement of thymidine incorporation to see if IPGs also stimulate proliferation of BeWo cells.

The procedure was similar.

An aliquot of $10^4$ cells in 100 µl of medium was plated in each well of a 96-well microtiter plate. After 24 h the medium was removed, the cells were washed twice with at least 100 µl of HBSS and 100 µl of serum free medium was added. Twenty-four hours later, the medium was withdrawn and the cells were stimulated with fetal calf serum, IPG preparations at different final concentrations or controls containing neither serum nor IPGs. Under these conditions, the cells were incubated for about 18-20 h, after which $[^3]$H]thymidine (1µCi/well) was added to each well for 4 h. At the end of this treatment, the medium was removed and the cells were trypsinised with 50 µl/ well for 10 min. The radioactivity incorporated in cellular DNA was then determined using a cell harvester and scintillation counter.
RESULTS

IPG Immunocytochemistry on chorionic villi sections of normal and pre-eclamptic placentae

Figure 1.2 shows the IPG Immunostaining using antibody 2D1 on villi frozen sections. Positive staining was detected in the trophoblast and internal stroma of both normal (a) and pre-eclamptic (b) villi compared to controls (c and d respectively). In pre-eclamptic sections (b), the internal stroma was generally more intensely stained than in normal villi (a) (n=3). Some of the cells in the stroma seem also to be strongly stained with the antibody in the pre-eclamptic placenta. These results confirm the presence of IPG in the placenta and support the observation that IPG levels are higher in pre-eclamptic placenta than in normal placenta.

Presence of IPG activity in both homogenates of placenta and in microvilli preparations

Cell proliferation assay

IPG A-and IPG P-type extracted from intact placenta and microvilli preparations of normal and pre-eclamptic placenta were active in stimulating the proliferation of EGFR T17 cells. Figure 1.3, 1.4 and 1.5 show the incorporation of thymidine into EGFR T17 cells after incubation of cells with medium only, fetal calf serum or different dilutions of IPGs. Figure 1.3 shows the incorporation of thymidine induced by a serial dilution of IPG A-and IPG P-type extracted from a homogenate of placenta. Both IPG A-and IPG P-type stimulate the proliferation in a dose dependent manner. Figure 1.4 indicates that IPG A-and IPG P-type extracted from microvilli also stimulate the proliferation of EGFR T17 cells in a dose dependent manner. IPGs extracted from microvilli of pre-eclamptic placentae were also active in stimulating the proliferation of the EGFR T17 cells. Figure 1.5 shows the dose response of thymidine incorporation induced by serial dilution of IPG P-type extracted from microvilli of both normal and pre-eclamptic placenta.

Table 1.3 displays the cell proliferation activities of IPGs obtained from different extractions, the ‘cpm values’ were normalised to ‘cpm control values’ (the control is cells with medium only). Thus, both IPG P-type and IPG A-type, extracted from homogenates of normal placenta, or from the microvillous membrane of normal and
pre-eclamptic placentae, stimulated the incorporation of thymidine into EGFR T17 cells. Every fraction of extracted IPGs was active in the cell proliferation assay, but the high variabilities amongst the activity of IPGs from different extractions resulted in no significant difference between IPG P-and IPG A-type as well as between IPGs from normal and pre-eclamptic placenta.

Table 1.4 shows the decrease in proliferative activity in the EGFR T17 cell assay of both IPG P- and IPG A-type after storage in solution at -20°C. Activity was measured for IPGs extracted from two different membrane preparations (Each extraction gave two fractions of IPGs: IPG A-type and IPG P-type). The same amount of IPG was assayed before and after storage, and as before the ‘cpm values’ were normalised to ‘cpm control values’ (the control is cells with medium only).

IPG P-type activity was found to decrease 2.5 and 2.9 fold. This decrease of activity is doubled for IPG A-type (5.2 and 6.2). Because of this instability observed when IPGs were kept in solution at -20°C, IPGs were stored at -80°C after freeze-drying. IPGs extracted from human placentae were previously shown to be stable for at least 6 months when stored at -80°C (personal communication from Dr S Kunjara, University College London). The greater instability of IPG A-type compare to IPG P-type may reflect in vivo stability.

cAMP dependent protein kinase A assay

Microvillous IPGs were also active in the modulation of enzyme activity. IPG A-type inhibited cAMP-dependent protein kinase A. Figure 1.6 shows the dose response of IPG A-type, extracted from microvilli of normal placenta, on inhibition of the enzyme. As seen 5 µl of the IPG A-type solution induced 68 % inhibition of cAMP dependent PKA, half of this volume induced a 37 % inhibition of the enzyme and the response is just detectable with 1.25 µl of the IPG A-type solution.

PDH phosphatase assay

IPG P-type extracted from microvilli preparations stimulated PDH phosphatase. Figure 1.7 shows the stimulation of PDH phosphatase induced by two samples of IPG P-type, IPG P-type extracted from microvilli of a normal placenta and IPG P-type extracted from microvilli of a pre-eclamptic placenta. As seen, IPG P-type extracted from microvilli of normal and pre-eclamptic placenta induced a 257 % and...
a 402% activation of PDH phosphatase respectively. Thus, whereas no significant
difference between normal and pre-eclamptic IPG P-type was observed by measuring
cell proliferation, PDH phosphatase stimulation was greater when incubated with
IPG P-type extracted from the microvilli of pre-eclamptic placenta. These results
have been determined for IPGs extracted the same day from the same amount of
starting material (calculated in protein content) in triplicate.
This result was obtained with material coming from only one pre-eclamptic placenta.
It can therefore not be concluded that pre-eclamptic microvilli membranes contain
higher IPG P-type activity compared to normal microvilli membranes. It should be
noted that the aim of the thesis was not to investigate differences between IPG
activity from normal and pre-eclamptic placentae. However, the finding is consistent
with the report of Kunjara et al (Kunjara et al 2000a) that shows that placental and
urinary levels of IPG-P type activity are elevated in pre-eclampsia.

**IPGs and proliferation of BeWo cells**
The proliferation of BeWo cells was measured in the presence of IPG A- and P-type.
**Figure 1.8** shows that serial dilution of microvillous IPG A-and IPG P-type did not
stimulate the incorporation of thymidine into BeWo cells. Whereas the presence of
fetal calf serum in the medium increased the proliferation more than four fold
compared to medium only, the presence of microvillous IPG A- and IPG P-type did
not significantly modify the incorporation of thymidine. Thus, whereas IPGs
stimulate the incorporation of thymidine into EGFR T17 cells, a similar proliferative
effect was not exhibited by BeWo cells. The experiment was carried under the same
conditions as the EGFR T17 cell proliferation assay (same number of cells, same
amount of IPGs, same incubation time).

**Active placental IPGs did not modulate leptin release from BeWo cells**
Leptin was measured in the medium of cultured BeWo cells in order to detect an
effect of IPGs in the modulation of leptin release by these cells. **Figure 1.9** shows
that leptin was released by BeWo cells in the medium. The unknown concentration
of leptin is determined in the RIA assay by measuring how much of a known amount
of the radioactively labelled hormone, $^{125}$I-human leptin, binds to a fixed quantity of
anti-leptin antibody in the presence of leptin. Thus, the difference in counts per
minute measured between the fresh medium (column 1) and medium after incubation
with cells (column 2) allows determination of the amount of leptin released. $1.2 \times 10^6$ cells produced approximately 1 ng of leptin in 5 hours (0.82 ± 0.08 ng in the experiment presented in Figure 1.9 and 0.90 in another experiment). Figure 1.9 also indicates that IPGs at 4 units/ml (one unit being the amount of IPGs inducing 50% of inhibition of PKA) and insulin at 10 nM did not modify the release of leptin by BeWo cells. In the presence of IPG A-type (column 3), IPG P-type (column 4), and insulin (column 5), the amount of leptin detected was 0.73 ± 0.15, 0.97 ± 0.20 and 0.82 ± 0.06 ng respectively.
Table 1.3: Activities of IPGs obtained from different extractions

<table>
<thead>
<tr>
<th>Normal Intact Placenta</th>
<th>IPG A-type</th>
<th>IPG P-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction 1</td>
<td>68 ± 21 / mg wet weight (n=3)</td>
<td>75 ± 26 / mg wet weight (n=3)</td>
</tr>
<tr>
<td>Normal microvillous membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extraction 2</td>
<td>89 760 ± 28 720 / mg protein (n=3)</td>
<td>63 840 ± 21 440 / mg protein (n=3)</td>
</tr>
<tr>
<td>Extraction 3</td>
<td>51 440 ± 9 200 / mg protein (n=3)</td>
<td>57 440 ± 16 240 / mg protein (n=3)</td>
</tr>
<tr>
<td>Extraction 4</td>
<td>64 960 ± 8 160 / mg protein (n=3)</td>
<td>80 240 ± 3 200 / mg protein (n=3)</td>
</tr>
<tr>
<td>IPG P-type extracted from microvillous membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>7 808 ± 2 080 / mg protein (n=3)</td>
<td>10 752 ± 2 720 / mg protein (n=3)</td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are cpm which represent the levels of incorporation of thymidine measured into EGFR T17 cells incubated with IPGs at a final dilution of 1/40. They were normalised to a value of control (no serum or IPGs added) of 100 cpm. IPGs have been extracted as described in Materials and Methods. In extraction 1, IPGs have been extracted from 1 g of placenta, in extraction 2, 3 and 4, IPGs have been extracted from a microvillous membranes preparation containing 0.4 mg of protein and in extraction 5, IPGs have been extracted from a microvillous membrane preparation containing 1 mg of protein. Values are means ± SD of triplicate determinations.
Table 1.4: Decrease of IPG activity within one week (cell proliferation assay)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Thymidine incorporation at time T (cpm)</th>
<th>Thymidine incorporation at time T+1week (cpm)</th>
<th>Ratio of thymidine incorporation values: T/T+1w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>IPG P-type</td>
<td>9013</td>
<td>3636</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>IPG A-type</td>
<td>7624</td>
<td>1464</td>
<td>5.2</td>
</tr>
<tr>
<td>Sample 2</td>
<td>IPG P-type</td>
<td>4474</td>
<td>1519</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>IPG A-type</td>
<td>14938</td>
<td>2418</td>
<td>6.2</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 1.2: Immunohistochemical staining of IPG in villi sections of normal and pre-eclamptic placenta. Frozen villi section of a normal placenta (a, c) and a pre-eclamptic placenta (b, d) were incubated in the presence of 2D1, an anti IPG antibody (a, b) or a non immune antibody (c, d). Bar represent 50 μm.
Figure 1.3: Incorporation of $^3$Hthymidine induced by serial dilutions of IPG A-type and IPG P-type extracted from intact placenta. Incorporation of thymidine was measured into EGFR T17 cells which were incubated in the absence of serum (c: control), 10% fetal calf serum (fcs, positive control), or serial dilutions of IPG A-type and IPG P-type which were extracted from 1g of placenta (final dilution of 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280). Values are means ± SD of triplicate determinations.
Figure 1.4: Incorporation of $[^3]$H thymidine induced by serial dilutions of IPG A-type and IPG P-type extracted from microvilli of normal placenta. Incorporation of thymidine was measured into EGFR T17 cells which were incubated in the absence of serum (c: control), 10% fetal calf serum (fcs, positive control), or serial dilutions of IPG A-type and IPG P-type which were extracted from a microvillous membrane preparation containing 0.4 mg of protein (final dilution of 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280). Values are means ± SD of triplicate determinations.
Figure 1.5: Incorporation of [³H]thymidine induced by serial dilutions of IPG P-type extracted from microvilli preparations of a normal placenta and a pre-eclamptic placenta. Incorporation of thymidine was measured into EGFR T17 cells which were incubated in the absence of serum (c: control), 10 % fetal calf serum (fcs, positive control), or serial dilutions of IPG P-type which were extracted from microvillous membrane preparations containing 1 mg of protein (final dilution of 1/40, 1/80, 1/160). Values are means ± SD of triplicate determinations.
**Figure 1.6**: Inhibition of cAMP-dependent protein kinase A induced by increased amounts of IPG A-type. IPG A-type was extracted from a microvillous membrane preparation containing 0.4 mg of protein. The extract, eluted with HCl at pH 1.3 (IPG A-type) was resuspended in 80 μl of distilled water before the assay.
Figure 1.7: Stimulation of pyruvate dehydrogenase phosphatase induced by extracts of IPG P-type from normal and pre-eclamptic microvillous membrane preparations. IPG P-type was extracted from microvillous membrane preparations containing 1 mg of protein. The extracts, eluted with HCl at pH 2 (IPG P-type), were freeze-dried and were resuspended in 80 µl of distilled water before PDH assay. A volume of 5 µl was used for the PDH activity determination. Each column is the mean value of triplicate estimations; the results are given as percentage of activation of that observed in the absence of IPG.
Figure 1.8: Incorporation of $[^3\text{H}]$thymidine into BeWo cells in the presence of serial dilutions of IPG A-type and IPG P-type. Incorporation of thymidine was measured into $10^6$ cells which were incubated for 20 h in medium without serum (c, control), in medium with 10 % fetal calf serum (fcs, positive control) or in medium containing serial dilutions of IPG A-type and IPG P-type extracted from a microvillous membrane preparation containing 1 mg of protein (final dilution 1/40, 1/80, 1/160, 1/320). Values are means ± SD of triplicate determination.
Figure 1.9: Determination of leptin released by BeWo cells. The amount of leptin was measured by RIA in fresh medium (column 1, negative control) and in medium after 5h incubation of 1.2×10⁶ BeWo cells in absence (column 2, control cell) and presence of IPG A-type (column 3), IPG P-type (column 4) or insulin (column 5). Values are means ± SD of triplicate determination.
DISCUSSION

The presence of IPGs in normal and pre-eclamptic placentae and more specifically in microvilli has been established by this work. The extracted IPGs, both A and P type, are biologically active in the cell proliferation and cAMP dependent PKA assays, but did not modify placental leptin release.

*Microvillous IPG A- and IPG P-type*

In the present work, IPG A- and IPG P-type have been extracted, following a method used to extract IPGs from homogenates of human placenta (Kunjara et al 2000a) and rat, bovine (Nestler et al 1991) and human liver (Caro et al 1997). The placental IPGs showed similar biological activities to IPGs from liver. They were able to modify enzyme activities (PDH phosphatase and cAMP-dependent PKA) and to stimulate proliferation of EGFR T17 cells. This suggests that the obtained IPGs are the same or similar compounds. A similarity in structure between the IPGs purified from different species was already proposed as human IPGs are active with enzymes and cells from other species (bovine, rat). This similarity has also been found between IPGs from different organs.

By analogy with IPGs extracted from human liver, placental IPGs are unlikely to contain protein or peptide like substances since Pronase E treatment does not suppress IPG activity on cell proliferation. They should contain phosphate, free amino groups and carbohydrates (Caro et al 1997). The carbohydrate content is supported by behaviour in the cellulose column. IPGs were retained on cellulose and eluted with acid but not with butanol. The fraction eluting on anion exchange resin with HCl at pH 2 has been referred as a solution of IPG P-type which contains chiro-inositol (or pinitol), and stimulates PDH phosphatase. This effect has been confirmed in our work. The fraction eluting with HCl at pH 1.3 has been categorised as IPG A-type, it contains myo-inositol, and is active on cAMP dependent PKA. This effect of this fraction has also been confirmed.

These water-soluble compounds were found to have different levels of stability when kept one week at -20°C in solution. Both IPG A- and IPG P-type activities decreased, with IPG A-type activity decreasing more rapidly than IPG P-type activity. This supports other differences between the two mediators. Maybe IPG P-type is also
more stable in physiological conditions and mediates more long term effects compared to IPG A-type, although as yet there is no additional evidence to support this hypothesis.

**IPGs and leptin release**

IPGs are both autocrine and paracrine factors. In addition to the two effects of IPGs proposed in the human placenta, stimulation of the steroidogenesis (Nestler et al 1991) and glycogenesis (Kunjara et al 2000a), we investigated the effect of IPG on placental leptin release. As IPG A-type modulates leptin release from rat adipocyte cells, and because leptin is a peptide not only made by adipocyte tissue but also by the placenta, we suspected a similar effect of placental IPGs on the regulation of placental leptin release. We used BeWo cells, a placental cell line, which have previously been shown to produce leptin (Masuzaki et al 1997). In the last report, in one day 2×10^5 BeWo cells produced approximately 1 ng of leptin. We obtained approximately 1 ng (exactly 0.82 and 0.90 ng in two different experiments) of leptin produced by 1.2×10^6 plated cells in five hours. (The same group also determined the production of leptin by cultured amnion cells of 5ng/10^6 cells per 5 days). Our results showed that the release of leptin by BeWo cells was not modified by placental IPGs. These IPGs inhibited cAMP-dependent protein kinase A, stimulated cell proliferation of EGFR T17 cells and did not stimulate the proliferation of BeWo cells. The amount of IPGs used was comparable to that used by Kunjara et al (Kunjara et al 2000b), therefore at physiological concentrations it appears that IPGs do not affect placental leptin release.

In adipocytes, inhibition of leptin release by IPGs occurred within 1 hour. The rapidity of the effect suggests a direct effect at the level of transcription. A transcriptional effect of IPGs has already been shown in isolated rat hepatocytes. IPGs decreased the rate of transcription of the gene for phosphoenolpyruvate carboxykinase enzyme and increased the rate of transcription of the α2-microglobulin gene (Alvarez et al 1991a). Another group also showed a transcriptional effect of IPGs on the p33 gene in rat hepatoma cells (Sato et al 1988a, Sato et al 1988b). However, the regulation of human leptin gene transcription has been shown to differ between placental trophoblasts and adipocytes (Ebihara et al 1997). The investigation of the human leptin 5'-flanking sequences in BeWo and
adipocyte cells showed that deletion between -1885 and -1830 of promoter sequences (upstream of the transcription start site) inhibited transcription in BeWo cells whereas identical deletion had no effect on the transcription of the leptin gene in adipocytes. This may explain the difference in response between adipocytes and BeWo cells towards IPGs, and suggests as a consequence that the IPG effect on leptin release may be cell type specific, i.e., require interaction with transcription factors found in adipocytes but not expressed in the placenta.

It should be also noted that extrapolation of the leptin data in BeWo cells to normal may not be reliable since BeWo cells are choriocarcinoma cells and therefore even used as a model for placental function differ from trophoblast cells.

**IPG location in the placenta**

The presence of IPGs in the placenta was demonstrated in two ways in this study: firstly, by immunostaining and secondly by chemical extraction followed by IPG-like activity assessment. These findings provided information about the precise cellular localisation, thus completing work started by Kunjara et al (Kunjara et al 2000a), in which IPGs were extracted from homogenates of placenta, and Suzuki et al (Suzuki et al 1984), in which PDH phosphatase active IPGs were found in the plasma membrane isolated from human term placenta.

The trophoblastic cytoplasm and internal stroma of placental villi were positively stained by the anti-IPG antibody. The staining was diffuse and not specific to a cellular type. The results were not surprising as IPGs have been found in numerous tissues (Kunjara et al 1995), and have therefore been described as ubiquitous factors. Cytoplasmic location of IPGs is in accord with the accepted mechanism of release of IPGs, in which IPGs are first released in the extracellular medium and then enter the cell where they exert their biological effects. In placenta IPGs were proposed to activate glycogen synthase (Kunjara et al 2000a), to suppress aromatase activity (Nestler et al 1991), and to stimulate 3β-hydroxysteroid dehydrogenase (Nestler et al 1991). The intracellular location of these enzymes supports the presence of IPGs inside the cell.

IPGs were also found to be present in microvilli preparations of human term placentae. They were extracted using a similar method to the one used for homogenates of placenta, which was adapted for microvilli preparations.
Microvillous IPG A- and P type stimulated the proliferation of EGFR T17 cells and inhibited cAMP dependent PKA, and microvillous IPG-P type stimulated PDH phosphatase. The presence of IPGs in both membrane and cytoplasm raised the question of the origin of the IPGs extracted from homogenates of placenta.

The presence of IPGs in the brush-border membrane was not observed by immunostaining. An explanation is that the antibody is raised against an IPG form corresponding to the form present in the cytoplasm. This would imply that IPGs of microvilli, not recognised by the antibody, have a different structure or are associated with other compounds in such a way that they are not accessible to the antibody.

It should be noted that several anti IPGs antibodies were tested. The results presented are the ones where I had reproducible observations. The group from Spain who provided these antibodies obtained interesting observations with another antibody (A1). A1 did not stain microvilli but did stain the basal membrane of chorionic villi sections. Obviously, these anti-IPG monoclonal antibodies do not recognise the same epitope. They may recognise different subtypes of IPGs or as mentioned above, an unrecognised associated IPG form. It should be also noted that the sections are different. Whereas the Spain group used paraffin sections (in which the tissue was fixed before immunostaining), in this work frozen sections in which fixation was done after immunostaining were used.

Different processes are used to obtain IPGs mediators. They include direct extraction methods (Nestler et al 1991, Kunjara et al 1995, Caro et al 1997, Kunjara et al 1999, Kunjara et al 2000a, and present work), release of IPGs into medium following growth factor stimulation of intact cells (Macaulay and Larkins 1988), and release of IPGs after treatment of membrane or purified glycolipid with PI-PLC enzymes (Alvarez et al 1991a). In the placenta, in addition to the direct extraction methods (Kunjara et al 2000a), IPGs have also been obtained by treatment of placental membrane following insulin stimulation (Suzuki et al 1984). The insulin mediators were obtained by formic acid extraction. The molecular weight was determined as 1500 Da. The insulin mediators obtained after formic acid extraction were found to bind to the ion exchange resin AG1X8. The compounds were eluted using NaCl (0-0.4M). We also used the ion exchange resin AG1X8, but we obtained IPG P-type by elution with HCl at pH 2.0 (0.01M) and IPG A-type by elution with HCl at pH 1.3 (0.05M). Their mediators stimulated PDH activity and inhibited glucose-6-
phosphatase activity. They were released upon insulin stimulation in a dose dependent manner. For higher concentrations of insulin or at high concentrations of extracted mediators, the activity declined. It seems that their IPG fraction contains the two subtypes IPG A- and P type. IPG P-type by definition should stimulate PDH activity. It is possible that IPG A-type presence explains the inhibitory effect observed at high concentrations, since Kunjara et al reported an antagonistic effect of IPG A-type towards PDH phosphatase (Kunjara et al 1999). In the present study, IPGs were isolated without insulin stimulation of the membrane. In Suzuki’s report, PDH activity is lower but also present in the absence of insulin stimulation. The plasma membrane fraction they used might include the syncytial brush-border membrane, syncytial basal plasma membrane and non-trophoblastic cell plasma membrane. In our work, the extracted IPGs originate precisely from microvilli of the syncytial brush-border plasma membrane.

IPGs have been extracted from microvilli preparations. Because they are water-soluble compounds (hydrophilic), they should not be components of the membrane. Therefore they might be associated with membrane lipid. On the one hand, if we consider a covalent binding of IPGs with a lipid, the molecule under consideration would be a glycolipid, a glycosylphosphatidylinositol (GPI), in other words the precursor molecule of IPGs. Our extracted molecule differs from GPI. Firstly GPI should not be water-soluble, secondly IPGs were not stained by iodine (data not shown) and finally, extracted IPGs are retained in cellulose columns and eluted with acid but not with butanol or butanol:ethanol (4:1:1) which should elute lipid. However, it is possible that IPGs were released from GPI during the extraction procedure, perhaps during the acid and heat step. The finding, presented in Chapter 2, that GPI is not present or is undetectable in pre-eclamptic placenta whereas both IPG A- and P type were present and IPG P-type was more active in pre-eclamptic placenta does not support this explanation. On the other hand, non-covalent binding to membrane compounds could be considered. IPGs are polar compounds containing phosphate and zinc (IPG A-type) or manganese (IPG P-type). Ionic interactions may occur between IPGs and components of the membrane. Furthermore, an acylation of the inositol ring of these microvillous IPGs has been suggested by mass spectrophotometric analysis (preliminary result of our group). This acyl tail may provide a hydrophobic anchor to localise IPGs in the membrane.
Caveolae

Microvilli have been characterised as tubular caveolae by Anderson (Anderson 1998) based on their chemical composition (richness in cholesterol and GPI-anchored proteins). Caveolae are not clearly defined as stated p19, but in all cases they are plasma membrane parts with different biochemical compositions and physical properties of the rest of the plasma membrane. Caveolae were reported by Ockleford (Ockleford and Whyte 1977) to be present in the human placenta, they were described as invaginations at the basis of the microvilli, which are strongly stained by Ruthenium red and Alcian blue. As Alcian blue stains glycosaminoglycans, IPGs and/or GPI may be stained. These observations may provide additional information about the precise location of IPGs in microvilli, suggesting an IPG/GPI localisation at the basis of microvilli. The mechanisms described in caveolae of other cellular types may exist in our ‘syncytial caveolae’, the microvilli.

Caveolae are considered as a focal point for insulin signal generation (Stralfors 1997) for the following reasons. First, IPGs precursors seem to be localised in caveolae (Parpal et al 1995). Second, glucose transporters are translocated to caveolae for glucose transport in response to insulin (Gustavsson et al 1996). Third, activated/autophosphorylated insulin receptors are found in caveolae and finally disruption of caveolae integrity eliminates protein phosphorylation in response to insulin signalling. The presence of an IPG transporter in caveolae has also been suggested by Varela-Nieto et al (Varela-Nieto et al 1996) bearing in mind that the IPG transporter needs to be localised near the site of release to permit the autocrine effect to occur and to avoid a scattered release of IPGs in the extracellular medium. To resume, insulin signal transduction using IPGs as second messengers, described in caveolae of other cellular types, might exist in placental microvilli, which are caveolae-like structures.

In chapter 2, further comparisons between placental microvilli and caveolae of other cellular types will be presented. These comparisons concern caveolae shapes which are related to lipid composition.

IPGs and signal transduction in the placenta

IPGs are messengers involved in a novel pathway of signal transduction which, as already suggested by comparison with caveolae of other cellular types, might be present in the placenta.
Several reports have presented evidence implicating IPG/GPI in the transduction signal of insulin (review in (Varela-Nieto et al 1996, Jones and Varela-Nieto 1998)). The breakdown of GPI was found to be correlated with insulin receptor levels (Macaulay et al 1992). Another group used mutant cells unable to make GPI. The cells responded to insulin by tyrosine phosphorylation, but without downstream metabolic effects (Lazar et al 1994). It was also reported that cells with kinase deficient insulin receptors do not hydrolyse GPI following insulin stimulation (Villalba et al 1990).

Insulin receptors are present in placental microvilli (Nelson et al 1978, Whitsett and Lessard 1978, Whitsett et al 1979, Jones et al 1993). Bovine liver IPG A- and P type were shown to stimulate 3β-hydroxysteroid dehydrogenase and suppress aromatase activity in human cytotrophoblasts, suggesting that IPGs mediate the insulin effect of steroid biosynthesis in this organ (Nestler et al 1991). The presence of both IPGs and insulin receptors in the human placenta, supports the hypothesis of the existence of a signal transduction pathway involving IPG release/GPI hydrolysis in the human placenta.

As mentioned earlier, caveolae were involved and proposed to be the site of IPG signal transduction in other cellular types. Based on Ockleford and Whyte’s (Ockleford and Whyte 1977) observation (IPG/GPI at basis of microvilli), a model can be proposed in which the insulin receptor, present throughout the entire surface of the microvilli, moves to the base of microvilli once bound to insulin. In this invaginating part of the membrane, the activated receptor would release IPGs which mediate some of the downstream effects of insulin.

This signal transduction pathway is not insulin specific. It seems to be used by other hormones or growth factors since GPI hydrolysis has been observed after stimulation of the factors listed in Table 1.2. Among them, IGF-I, prolactin and human chorionic gonadotrophin (hCG) are hormones which have important functional roles in the human placenta (Spellacy et al 1967, Simpson and MacDonald 1981, Siler-Khodr et al 1991, Gluckman and Harding 1997, Muyan and Boime 1997, Bauer et al 1998, Petraglia et al 1998). TGFβ was also found to be involved in differentiation of the trophoblast and may have a role in pre-eclampsia (Caniggia et al 2000).
**IPGs and pre-eclampsia**

Defects in the IPG signalling system have been reported in pathological conditions, such as diabetes and obesity, and in conditions of altered metabolism such as ageing. In pre-eclampsia, a difference between normal and pre-eclamptic IPG P-type activity was observed (Kunjara *et al* 2000a). Higher levels of IPG P-type activity were found in urine and placental samples of pre-eclamptic women. Similar results were found when IPGs were extracted from the microvillous membrane. In addition, the immunostaining experiments indicated a higher intensity of staining in pre-eclamptic sections, suggesting that higher activity is due to a higher amount of IPGs rather than a different structure.

As proposed by Kunjara *et al* (Kunjara *et al* 2000a) and illustrated in Figure 6, the high IPG P-type activity explains the excess glycogen found in pre-eclamptic placenta. However, this role in glycogenesis does not explain the pathology of pre-eclampsia, since glycogen excess is also found in the placenta of diabetic pregnant women, which do not suffer from the pre-eclamptic disorder.

High IPG P-type activity, in both placenta and urine samples of pre-eclamptic pregnant women suggests that urinary IPGs originate from the placenta. Therefore IPGs should be present in the circulation. There are two possibilities, either IPGs circulate free, or they are associated with other serum components.

As pregnancy is associated with shed syncytial plasma membrane (Knight *et al* 1998) and the present work showed that IPGs have been extracted from preparations of the syncytial plasma membrane, we can hypothesise that IPGs circulate in blood, associated with microvilli.

Since this shedding is amplified in pre-eclampsia and syncytial membrane particles, isolated from human placenta inhibit the proliferation of human umbilical vein endothelial cells, it was postulated that this high shedding could account for the endothelial dysfunction characteristic of pre-eclampsia (Knight *et al* 1998). The same group then isolated, from the microvillous membrane, a complex of proteins, which have the same inhibitory effect on the endothelial cells. The complex contains integrins α₃ and α₅, dipeptidyl peptidase IV, α-actinin, transferrin, transferrin receptor, placental alkaline phosphatase and monoamine oxidase A (Kertesz *et al* 1999). It would be interesting to investigate the presence of IPGs in this complex.
Pre-eclampsia: a defect of IPG/GPI signal transduction?

The shedding of microvilli, which is amplified in pre-eclampsia, may result in higher circulating IPGs which would explain the higher IPG activity in the urine of pre-eclamptic women. However the question of higher levels of IPGs in the placenta remains unresolved. This could be the result of a defect in the signalling system using GPI hydrolysis which thus results in higher IPG release. This mechanism combined with increased microvilli shedding would explain the higher IPG P-type activity found in pre-eclampsia.

The precise mechanism of release of IPGs in the placenta remains to be elucidated. The generally accepted model, presented in Figure 1.1, implies an activation of a specific phospholipase after receptor ligation of insulin or other growth factors. The specific phospholipase hydrolyses glycosylphosphatidylinositol generating IPGs in the extracellular medium. IPGs then enter the cell where they exert their biological effects.

Figure 1.10 represents what is known and is not known about the IPG signal transduction in normal and pre-eclamptic placentae and more precisely around the microvillous brush-border membrane. In our model, the plasma membrane is the syncytial microvillous membrane, the extracellular medium is the maternal blood space and the intracellular medium is the syncytiotrophoblast cytoplasm. We know that IPGs are present in the placenta and that IPGs are found more precisely in the cytoplasm as well as in microvilli. IPG P-type activity is higher in pre-eclamptic placenta. Nothing in the literature concerns the glycolipid precursor molecule of IPGs, GPI. In chapter 2, the presence of GPI in normal and pre-eclamptic placenta, and more precisely in microvilli preparations has been investigated. The possible mammalian enzyme responsible for the release of IPGs from GPI is GPI-PLD. The enzyme is present in the human blood and its activity in the placenta has been proposed. The presence of GPI-PLD in microvilli is not known, and there is no information in the literature concerning GPI-PLD and pre-eclampsia. An investigation of GPI-PLD in the placenta is the subject of Chapter 3. Thus, this schematic drawing will be completed in the following chapters.
Summary

In summary, the results presented in this Chapter indicate that IPGs are present in the human term placenta, more precisely in both the trophoblast cytoplasm and the syncytial microvillus membrane. These findings suggest that the machinery involved in the signal transduction of insulin and/or other factors through IPG release/GPI hydrolysis exists in this part of the placenta. In the following chapters, different components of this signalling system are studied in order to understand the high levels of IPG-P type found in pre-eclampsia.
Normal Pre-eclampsia

Figure 1.10: Hypothetical model of IPG release in the microvillous membrane of normal and pre-eclamptic placenta. IPG may be released from microvillous membrane after stimulation of a GPI-specific phospholipase, such as GPI-PLD, which would hydrolyse GPI to generate IPGs. A defect of the mechanism of IPG release may explain the higher level of IPGs or IPG P-type activity reported in pre-eclamptic placentae. Abbreviations: GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidyl-inositol-specific phospholipase D; IPG, inositolphosphoglycan; STB, syncytiotrophoblast.
Chapter 2

GPI in Placenta
INTRODUCTION

The IPG precursor molecule is a membrane linked IPG with a lipid tail, in other words, a glycolipid with the general structure lipid-phosphate-inositol-glycan, referred as glycosylphosphatidylinositol (GPI). The existence of the IPG precursor as a membrane glycolipid was suspected when PI-PLC was found to be as capable as insulin in generating IPGs from membranes. Saltiel and Cuatrecasas (Saltiel and Cuatrecasas 1986) and Mato et al (Mato et al 1987a) extracted this membrane glycolipid, which was cleaved by PI-PLC generating IPGs. The production of IPGs by PI-PLC indicated a phosphodiesterase cleavage of the precursor molecule, the first information about the nature of GPI.

Structure of the IPG precursor molecules

The polar head group: Since IPG structures have not yet been determined, it is axiomatic that the precise chemical structures of the IPG-precursor GPIs have also not been clearly defined. Thus, the diversity of chemical structures described for the soluble IPGs holds good for the polar head group of the membrane GPIs. In addition to multiple possible glycan structures on the polar side, several types of lipids can form the lipid moiety.

The lipid moiety: Myristate (C14) and palmitate (C16) were the two saturated fatty acids that could compose the lipid moiety of the IPG glycolipid precursors. Saltiel et al (Saltiel et al 1986) reported incorporation of [3H]myristate into glycolipid which was then found in diacylglycerol and phosphatidic acid, products of the degradation of GPIs after phospholipase hydrolysis. Mato’s group (Mato et al 1987a) purified the glycolipid precursors after the incorporation of various radiolabelled fatty acids in hepatoma cells. The principal incorporated lipid was [3H]palmitate, while myristic acid, stearic acid, linolenic acid and arachidonic acid were found to be significantly less important comprising less than 10% of palmitate incorporated. The molecule was cleaved by PLA₂ but resistant to mild alkali hydrolysis suggesting the presence of a 1-hexadecyl,2-palmitoyl-glyceryl moiety in the purified lipid.

The structure of GPI, lipid-phosphate-inositol-glycan has been described in several other molecules such as GPI-anchored proteins, glycoinositolphospholipids (GIPLs),
lipophosphoglycans (LPGs) and lipopeptidoglycans (LPPGs). In spite of no evidence of the release of IPGs from these molecules, they are presented in this introduction since they have been well characterised.

**IPG precursor related molecule: free GPI of mammalian cells**
Free GPI are glycosylphosphatidylinositol compounds which do not anchor attached proteins. The precursor molecules of IPGs are free GPI. Free GPI have been described in mammalian cells by van’t Hof et al, Baumann et al and Singh et al (van’t Hof et al 1995, Singh et al 1996, Baumann et al 2000). These groups have not investigated the relation of these free GPI with IPG. In these studies, some of the GPI extracted corresponds to well characterised GPI molecules, termed H6-H7-H8, which are structurally very similar to protein-bound GPls. Their structures are shown in Figure 2.1. They were all identified by the incorporation of [3H]mannose or [3H]ethanolamine. In the van’t Hof study (van’t Hof et al 1995), free GPI from Madin Darby canine kidney cells were identified by their sensitivity to GPI-PLD and nitrous acid. They were PI-PLC insensitive, presumably due to the presence of an acyl chain on the inositol ring. Singh et al (Singh et al 1996) described free GPI extracted from a human carcinoma and mouse lymphoma cell line. The lipid moiety was identified as a diacylglycerophosphoinositol; the glycerol backbone was found to be distinct from the GPI protein anchors of the same cells and did not undergo the lipid-remodeling reaction characteristic of protein-linked anchors.

Gaulton and Pratt (Gaulton and Pratt 1994) also reported the presence of different free GPI subtypes in T lymphocytes detected with radiolabelled antibody reactive with GPI. Upon insulin stimulation, some of the GPI were no longer detected suggesting that only a subset of GPI is involved in insulin signalling and thus that the different subtypes served different functions.

**IPG precursor related molecules: GPI-anchored proteins**
GPI-anchored proteins, proteins linked to the membrane by GPI, are found in several species. They contain an identical Ethanolamine-PO₄-Mana₁-2Manα₁-6Manα₁-4GlcNα₁-6myo-inositol backbone linked to a lipid. Figure 2.2 details the structures of known mammalian GPI-anchored proteins. Among the mammalian GPI-anchored proteins, those for which the GPI structures has been characterised include: rat brain
Thy-1 antigen (Homans et al 1988); human erythrocyte acetylcholinesterase (Deeg et al 1992, Roberts et al 1988); hamster brain scrapie prion protein (Stahl et al 1992); bovine liver 5'-nucleotidase (Taguchi et al 1994); human placental alkaline phosphatase (Redman et al 1994); human urine CD59 (Nakano et al 1994); mouse skeletal muscle neural cell adhesion molecule (NCAM) (Mukasa et al 1995); human spleen CD52 (Treumann et al 1995) and porcine and human renal membrane dipeptidase (Brewis et al 1995). The structures of the GPI anchors of GPI-anchored proteins in parasitic protozoa, yeast and fish have also been characterised (McConville and Ferguson 1993).

In all GPI-anchored proteins, the glycolipid is attached through the ethanolamine (EtN) moiety to the carboxy terminus of the protein by an amide bond (Kodukula et al 1993, Moran and Caras 1994). The diversity within GPI anchors is mainly reflected in the location and nature of the branching groups of the glycan (McConville and Ferguson 1993, Stevens 1995). The lipid residue of many of the GPI anchor structures is a diacylglycerol moiety, but alkylacylglycerol residues are not uncommon, and ceramide structures have also been identified (Hung et al 1995, Ko et al 1995). Ceramide is the most common lipid in the anchors of yeast (McConville and Ferguson 1993). Lyso-1-O-stearoylglycerol is also found in GPI structures of Leishmania proteins (McConville and Ferguson 1993).

It is noteworthy that GPI-anchored proteins, which are attached to the external face of the plasma membrane, may have different GPI. Some proteins possess a certain degree of micro-heterogeneity in the structure of their GPI carbohydrate side chain. For example, it was reported that all GPI anchors of rat brain Thy-1 contain a N-acetylated galactosamine residue but only 71% of them contain a fourth mannose residue (Homans et al 1988).

The structure-function relationship between GPI-anchored protein and the GPI which serves as the IPG-precursor molecule remains unknown. Similarities and differences exist between them.

On the one hand, both IPG precursor glycolipid and GPI-anchored proteins share a susceptibility to cleavage by PI-PLC. However, an additional fatty acid on the inositol ring renders the molecule resistant to the enzyme, for example palmitoylation of the myo-inositol at the 2'-hydroxyl renders certain GPI anchors resistant to PI-PLC hydrolysis (in review (Varela-Nieto et al 1996). This resistance
to cleavage of the GPI by PI-phospholipase C also occurs with the free GPI isolated by van’t Hof (see above). In addition, IPG precursor glycolipid and GPI-anchored protein share a sensitivity to nitrous acid deamination, the presence of saturated fatty acids (palmitate and myristate mainly) in the diacylglycerol moieties, and cross-reactions of antibodies. On the other hand, differences between IPG precursor GPI and the GPI of GPI-anchored proteins exist in the complex glycan moiety. The presence of mannose has not been shown in IPG precursor GPI. Furthermore, whereas only diglyceride was detected in free GPI (Saltiel et al 1986, Mato et al 1987a), diglyceride, monoglyceride and ceramide-like residues have been found in the phosphatidylinositol of GPI anchors (McConville and Ferguson 1993).

The hypothesis that GPIs of GPI-anchored proteins are IPG precursor molecules has been discussed in several reports (Gaulton and Pratt 1994, Rademacher et al 1994, Varela-Nieto et al 1996), but to date there is no evidence of the production of IPG second messengers from GPI-anchored proteins (Rademacher et al 1994). Nevertheless, studies with mutant cell lines (Avila et al 1992, Lazar et al 1994) affecting the biosynthesis of GPI-anchored proteins suggest that the free GPI (IPG precursor GPI) and GPI-protein anchors share the same biosynthetic pathway until a certain stage (Figure 2.3). Cell lines K562 IA and K562 IVD are erythroleukaemia cell lines deficient in the deacylation of PI-GlcNAC. These cells are deficient in GPI-anchored proteins and probably also deficient in IPG precursor GPI since they are not capable of synthesising glycogen upon insulin stimulation (Lazar et al 1994) (IPGs are involved in the signal transduction of insulin for glycogen synthesis (Larner et al 1979b, Huang et al 1993). The deficiency in both IPG precursor GPI and GPI-anchored proteins suggest that these molecules share the same biosynthetic pathway. Another cell line incapable of producing GPI-anchored proteins, MVB2 (mutant derived from T cell hybridoma), was shown to contain free GPI and was able to release active IPGs suggesting that these free GPI were IPG precursor molecules (Avila et al 1992). As schematised in Figure 2.3, these observations suggest that the common biosynthetic pathway of IPG precursor GPI and GPI of GPI-anchored proteins divide into two pathways, one leading to GPI-anchored protein, the other to IPG precursor GPI. Differences between the two molecules may reside in the glycan part since galactose but not mannose was found in IPG precursor GPI and GPI-anchored proteins contain mannose.
However free GPI which do contain mannose have also been reported (van’t Hof et al 1995, Singh et al 1996, Baumann et al 2000) and these may use a different biosynthetic pathway since free GPI described by Singh et al differs from GPI of GPI-anchored proteins of the same cells by the glycerol backbone.

**IPG precursor related molecules: GIPLs, LPGs and LPPGs**

GIPLs and LPGs are two distinct classes of GPI described in the parasitic protozoa *Leishmania*. (review in (McConville and Ferguson 1993)). They are metabolic end products containing the core sequence Manα1-4GlcNα1-6myo-inositol attached to a lipid. The GPI anchor of LPG is distinct from protein GPI anchors as it contains additional galactose residues and an additional Glc1P on one of the mannose residues of the core glycan. The lipid moiety is a monoglyceride since a lysalkyl-phosphatidyl inositol with either a C24:0 or C26:0 alkyl chain was described (Galα1-6Galα1-3Galβ1-3Manα1-3Manα1-4GlcN-lyso-Pl). LPGs are characterised by a size variable phosphoglycan chain of 4-40 kDa which is attached to the terminal Gal of the GPI core. The phosphoglycan moiety is made up of repeated units containing phosphate, galactose and mannose residues. The end of the phosphoglycan chain is capped by neutral oligosaccharides. It is noteworthy that phosphoglycan chains, lacking both the lipid and the core region of the LPG anchor, were found released into the culture medium.

The GIPLs are the major glycolipids of *Leishmania* parasites (Ralton and McConville 1998, Zawadzki et al 1998). These free GPs (free because GPs are not linked to proteins or phosphoglycan moieties) share features in common with either the protein and/or LPG anchors. The glycan headgroup of type 1, type 2 and the hybrid type, are structurally related respective to the GPI protein anchors, the LPG anchors and common features of both types of GPI anchor. The lipid moieties are alkylacyl PI, lysoalkyl-PI with alkyl chains of C18:0, C24:0 and C26:0 or diacylglycerol with palmitoyl, stearoyl and heptadecanoyl (Zawadzki et al 1998). GIPLs have also been described recently in the *Trypanosoma cruzi* epimastigote form (one life cycle stage). These molecules were absent from the trypomastigote form (another life cycle stage) of *T. cruzi* which contains other GPI molecules inducing release of nitric oxide and cytokines (Almeida et al 2000).
Another free GPI glycolipid has been described as a heterogeneous lipopeptidophosphoglycan (LPPG) in a kinetoplastid parasite, in *Trypanosoma cruzi*. The chemical structure is similar to the protein GPI anchors of the species, although it differs because of additional residues on the glycan core and a ceramide lipid moiety instead of alkylacylglycerol (McConville and Ferguson 1993).

The related structural and chemical features of these molecules with the IPG precursor glycolipid raises the question of their ability to be cleaved in order to generate IPG second messengers and/or the possibility of sharing their biosynthetic pathway.

**GPI cellular location**

IPG precursor glycolipid is present in the plasma membrane. As mentioned in the introduction of Chapter 1, the majority of the IPG precursor was found at the outer surface of the cell in rat adipocytes and hepatocytes (Alvarez *et al* 1988, Varela *et al* 1990). (It is part of the evidence for the extracellular IPG release.)

Previously mentioned GIPLs and LPGs described in *Leishmania* parasites were also found at the cell surface, where they are accessible to antibodies (McConville and Ferguson 1993). GIPLs have a relatively low turnover rate whereas LPGs are actively shed from the cell surface and have a high turnover.

The free GPI extracted from mammalian cells (Madine-Darby canine epithelial cells (MDCK), HeLa cells, Thy1+ mouse thymoma cells and ELT4 lymphoma) were mainly located at the cell surface (van’t Hof *et al* 1995, Singh *et al* 1996, Baumann *et al* 2000). The content of free GPI (potential anchors and metabolic end products) was estimated to range from $10^5-10^7$ molecules/cell in MDCK cells (van’t Hof *et al* 1995).

The presence of free GPI has been studied in the polarised epithelial MDCK cells, using viruses which assemble at the cell surface and acquire their envelope lipids from the plasma membrane (van’t Hof *et al* 1995). Free GPI were found to be present in the plasma membrane and did not display a differential distribution between the
apical and basolateral domain. This location pattern differs from GPI-anchored proteins which exist exclusively at the apical polar membrane domain.

These mammalian GPIs were proposed by the authors to be located in the cytoplasmic leaflet of the bilayer. Their argument took into consideration the fact that GPIs are synthesised in the cytoplasmic leaflet of the endoplasmic reticulum and that subsequent intracellular lipid transport would not change this orientation. They also argue that differences in lipid composition between the apical and basolateral plasma membrane are confined to the exoplasmic leaflet of the membrane bilayer and GPIs display a non-polarised distribution. The occurrence of a transbilayer movement (flip flop) was also mentioned by the authors.

Actually, the proposal for a location of free GPI in the cytoplasmic leaflet of the bilayer stated above has not been confirmed but in contrast, Singh et al (Singh et al 1996) and Baumann et al (Baumann et al 2000) provide experimental evidence of the presence of free GPIs in the exoplasmic (external) leaflet of the plasma membrane.

In the biosynthetic pathway, free GPIs are synthesised, by sequential addition of monosaccharides to phosphatidylinositol, in the endoplasmic reticulum. They use vesicular transport through the Golgi apparatus. And they arrive at the cell surface in a time and temperature dependent fashion (Singh et al 1996) where they are concentrated, although they only contribute to a minute fraction of bulk lipid amounts (Baumann et al 2000).

Reasons for investigating GPI in the normal and pre-eclamptic placenta

The presence of IPGs has been reported in the placenta (Chapter 1, (Suzuki et al 1984, Kunjara et al 2000a)). This suggests the presence of the glycolipid IPG precursor in the placenta although it has not been physically isolated.

The presence of IPGs in microvilli preparations, the established theory that IPGs are released from plasma membrane glycolipids and the location of GPI at the cell surface of mammalian cells, suggests that placental IPG precursor molecules are present in the microvilli membrane.

The defect in the IPG/GPI signalling system hypothesised to exist in pre-eclampsia (Chapter 1) may affect IPG precursor GPI, one of the key molecules of this signalling pathway.
Since high levels of IPGs were found in pre-eclamptic placenta, high levels of the precursor molecules were also expected.

Aim of the chapter
This chapter investigates the presence of the glycolipid GPI in human placenta. GPI has been extracted from total membrane preparations of placenta and more specifically from microvilli membrane preparations. The extraction was performed on both normal and pre-eclamptic samples.
Figure 2.1: Structures of H6, H7 and H8 (from references (van't Hof et al 1995, Singh et al 1996, Baumann et al 2000)).
Figure 2.2: Structures of known mammalian GPI anchors. The conserved core region contains an extra ethanolamine phosphate (EtNP) in all mammalian structures. Various side chain modifications of carbohydrate, additional EtNP, and/or palmitate (R₁-R₄) are found as indicated. In some proteins certain residues may only be present in a proportion of GPI anchor anchors, and this is indicated by ±. OH indicates that no modification is thought to be present. R₅ = lipid moiety present. nd = not determined. (From reference (Brewis et al 1995)).
Divergent biosynthetic pathways for protein-bound and free GPIs

Figure 2.3: Proposed divergent biosynthetic pathways for protein-bound and free GPIs (IPG-precursor GPI).
MATERIALS & METHODS

1- GPI Extraction

Materials

Placentae: Normal and pre-eclamptic placentae used to extract GPI were obtained from UCL Hospital. Pre-eclampsia was diagnosed by clinicians as detailed in Materials and Methods of Chapter 1. Placentae were collected quickly after delivery and freeze clamped after removing basal plate and chorionic plate.

Microvillous membrane preparations were the same preparations as those used in Chapter 1 for IPG extraction.

Silica powder was Silica Gel G (G or 60 0.040-0.063) purchased from BDH Chemicals Ltd (Poole, England). TLC plates and Iodine were also obtained from BDH Chemicals Ltd (Poole, England). Phosphatidic acid, phosphatidylecholine Anisaldehyde and orcinol were obtained from Sigma Aldrich Ltd (Poole, England).

Methods

GPI was isolated from total plasma membrane and microvillous membrane as schematised in Figure 2.4 following a method described previously (Caro et al 1997) with minor modifications.

Frozen placentae (15g) were reduced into powder with mortar under liquid nitrogen, and homogenised in 50 ml of 0.9% NaCl using an electrical homogeneiser (Voss Instruments Ltd, Maldon, Essex, England). After centrifugation for 5 min at 20 000 g at 4°C, the pellet was discarded and the supernatant centrifuged for 60 min at 100 000 g at 4°C to obtain a pellet of total plasma membrane. The supernatant was discarded and the pellet extracted with 25 ml of chloroform/methanol/hydrochloric acid (1:2:0.02 v/v/v) by stirring for 30 min. Chloroform (40 ml) and 0.1 M KCl (40 ml) were added and stirred for 30 min. The organic and aqueous phases were then separated by low speed centrifugation at 4°C (10 min 1000 g), the aqueous phase was re-extracted with 40 ml chloroform and the organic phases were pooled. The solution was dried using a Buchi rotovapor (Switzerland) and the residue kept at -20°C overnight.
The extract was re-suspended in 60 ml of chloroform and mixed with 5 g of Silica powder, which has been previously activated by heating 1 h at 110°C. Thus, activated silica will allow the binding of polar lipids and apolar lipids will be discarded. After the mix was poured in a column, silica was washed with 5 bed volumes (50 ml) of chloroform/methanol/hydrochloric acid (300:50:3 by volume) and polar lipids were removed from the silica with 20 ml of methanol and collected into a round bottom flask. The methanol extract was then dried. A second extraction was then performed to isolate GPI. The extract was suspended in 20 ml chloroform and mixed with 5g of silica powder. After stirring, the mixture was poured in a column and washed with chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5 by volume), GPI were extracted with methanol as before, dried and kept at -20°C.

The extract was then dissolved in 1 ml of chloroform/methanol (1:1), loaded onto a TLC silica plate and developed in chloroform/methanol/water/ammonia (9/6/0.5/0.35) with phosphatidic acid and phosphatidylcholine as markers. The plate was then stained with iodine vapour for 5-10 min.

To collect the detected GPI, the silica was scrapped off the plate and GPI was extracted twice with 3 ml of methanol each time. The solution was aliquoted in glass tubes, methanol was evaporated and GPI was kept at -20°C (Stable for no more than one month). The yield obtained was approximately 0.3 mg of GPI per g of placenta. In some experiments, GPI was identified on TLC plates not only by iodine staining, but also by anisaldehyde and orcinol staining. Anisaldehyde and orcinol are supposed to stain GPI since they recognise carbohydrate.

When GPI was isolated from microvillous membrane preparations, the extraction was carried out at the same time as an extraction from a pellet of total plasma membrane containing the same amount of protein. Protein content was determined as described in Chapter 1. Fifteen grams of placental tissue gave a fraction of total plasma membrane containing 30 mg of protein. Since the aim of extraction of GPI from microvillous membrane was not to produce GPI but to know whether or not GPI was present, the extraction was carried out using less material. Membrane preparations containing about 10 mg of protein were used as starting material and products used for the procedure were reduced proportionally.
2- Determination of phosphate content

Materials
Ammonium molybdate and ascorbic acid were obtained from Sigma Aldrich Ltd (Poole, England). Perchloric acid (70% solution) was purchased from BDH Ltd (Poole, England).

Method
The phosphate content of the extracts of GPI was determined using a method which measures inorganic phosphate after release of phosphate groups by perchloric acid treatment. Potassium dihydrogen phosphate (0-20 nmoles) was used as standard. Samples and standard were evaporated to dryness in an oven at 100°C. After evaporation, 90 µl of 70% perchloric acid was added to the tubes which were capped, vortexed and placed at 180°C for 45 min. After cooling at room temperature, distilled water (600 µl) was added followed by 50 µl of 5% ammonium molybdate and 50 µl of 10 % ascorbic acid. The colour reaction was achieved by heating the samples at 95°C for 15 min. After cooling at room temperature, the optical absorbance was measured at 650 nm using a microplate reader (Molecular Devices Thermomax microplate reader) in conjunction with Softmax version 2.35.

3- Determination of amino-group content

Materials
Fluorescamine and D-Glucosamine were purchased from Sigma Aldrich Ltd (Poole, England). Silica powder was obtained from BDH Ltd (Poole, England). Nonidet P-40 (NP-40) was obtained from ICN Biomedicals Inc (Aurora, Ohio, USA).

Method
Free amino groups were detected in GPI extracts using fluorescamine. Fluorescamine is a non-fluorescent molecule that reacts with primary amino groups to yield a product that is highly fluorescent under UV irradiation. This reaction is illustrated in Figure 2.5. The quantification was determined using D-glucosamine as a standard (0-100 nmole). Since a matrix effect occurs in this assay, the determination has been performed using an appropriate standard consisting of variable amounts of D-
**Figure 2.4:** Placental GPI isolation. GPI is isolated from microvillous membrane and total plasma membrane of placenta as described in Materials and Methods. After a first extraction of most of the lipids, polar lipids are isolated by silica adsorption. Two further chromatographies lead to GPI isolation.
glucosamine (0-100 nmoles) to which a constant amount of the GPI to be determined was added. Dry GPI was mixed with 1 ml of solutions of known concentration of glucosamine in 200 mM Na Borate, 0.05% NP-40, pH 9 with 1 ml of 1 mM fluorescamine prepared in dry acetone (acetone was dried by mixing with silica powder and filtering). This mixture was incubated in a water bath at 60°C for 30 minutes. After the reaction the product was measured by fluorescence using an excitation wavelength of 380 nm and an emission wavelength of 470 nm.

Figure 2.5: Scheme representing the conversion of a non-fluorescent fluorescamine into a fluorescent fluorescamine form by reaction with primary amino groups.
RESULTS

Isolation of GPI from total placental membrane of normal placentae
Lipid fractions containing GPI were isolated from total placental membrane of normal placentae. Figure 2.6.A depicts the TLC plate after iodine staining of one of these extractions. The photograph was taken before GPI was removed from the plate. The resolution front (Rf) of GPI was about 0.5, Rf of PA about 0.25 and Rf of PC about 0.8. As seen on the figure two bands were detected suggesting the presence of at least two glycolipids. In similarity to IPGs, GPI may constitute a family of compounds rather than a single molecule.

GPI was stained by both iodine and anisaldehyde. Figure 2.7.A shows GPI stained by iodine and Figure 2.7.B shows the same plate which has been stained by anisaldehyde after disappearance of iodine staining. GPI was also stained by orcinol (data not shown). Iodine staining confirms the lipidic nature of GPI, and anisaldehyde and orcinol stainings suggest the glycosidic nature of GPI.

Figure 2.7 also shows the purification step. Lane 1 shows the TLC of the extract obtained after the first chromatography, which in theory contains all polar lipids, and lane 2 shows the purified GPI. As seen, there were more bands in the first extract showing that some polar lipids have been discarded by the sequential chromatography.

Isolation of GPI from microvillous membrane of normal placentae
GPI was also extracted from microvillous membrane preparations. Protein content and alkaline phosphatase activity were determined in both the total membrane fraction and the microvillous membrane fraction. The extraction of GPI on the fractions was carried out simultaneously with membrane containing identical amount of protein. Alkaline phosphatase activity was three fold higher in microvillous membrane than in total plasma membrane as expected since alkaline phosphatase is used as marker for microvillous membrane. Figure 2.8 shows the TLC of GPI extracted from microvillous membrane (lane 2) and GPI extracted from total plasma membrane (lane 1). As seen, lanes 1 and 2 show an identical staining pattern suggesting that GPI is not only present in total plasma membrane but also in
microvillous membrane. It also suggests that the extracted products are identical in the two membrane fractions.

Absence or un-detectable amount of GPI in pre-eclamptic placentae
Attempts to extract GPI from pre-eclamptic placentae have been carried out. Figure 2.6.B shows TLC of a GPI extract from a pre-eclamptic placenta. As seen GPI was not detected in the membrane preparation of a pre-eclamptic placenta. The extraction was performed at the same time and under the same conditions as the extraction from the membrane preparation of normal placentae shown in Figure 2.6.A. This finding has been reproduced for three different pre-eclamptic placentae (total plasma membrane). Similarly, GPI was not detected in microvilli preparations of pre-eclamptic placenta whereas it was from microvilli of normal placenta. The extractions were performed at the same time using the same amount of starting material.

Amino-group and phosphate content of the GPI preparation
The amino-group and phosphate content of the GPI preparation obtained were checked and measured to confirm the nature of GPI.

The amino-group content was measured using fluorescamine. Fluorescamine reacts with primary amino groups yielding a fluorescent adduct as illustrated in Figure 2.5. As is found in several analytical assays, a matrix effect exists. To determine the exact value of NH$_2$ content in GPI, a correction is achieved by an appropriate standard. Figure 2.9 shows the fluorescence induced by both the glucosamine standard (square) and the glucosamine standard to which was added a constant amount of GPI (triangle).

The amount of NH$_2$ in GPI was 460 nmole NH$_2$/mg. If the molecular weight of GPI is arbitrarily chosen as 2000, there would be 0.92 mole NH$_2$ per mole GPI, the ratio 1:1 is quite reasonable.

The phosphate content has also been determined. In contrast to the previous assay, this assay has no matrix effect. This is shown in Figure 2.10 which indicates that addition of GPI to the standard results in a linear increase in phosphate absorbance.
Figure 2.11 shows that the phosphate content was proportionate to added GPI. Determination gives a value of 9.6 n mole inorganic phosphate /mg GPI. This gives 0.02 mole PO₄/mole GPI, and a ratio NH₂ /phosphate of about 50:1.

These values seem to be underestimated. It has been shown by Lance Turtle, University College London (personal communication), that when the phosphate determination was performed with a known amount of phosphatidylinositol, the amount of inorganic phosphate detected is 100 fold less than expected. This finding is illustrated in Figure 2.12, the figure indicates that 0.2 n moles of phosphate have an OD corresponding to the OD obtained with 20 n moles of phosphatidylinositol. This suggests that the measurement does not properly assess the levels of phosphate when phosphates are covalently linked, although in theory perchloric acid treatment should release phosphate. Because phosphatidylinositol and GPI have the inositol-phosphate structure in common, it seems obvious that the determination of phosphate in GPI by this method is like that for phosphatidylinositol, ie underestimated. Assuming that GPI behaves similarly to phosphatidylinositol, our determination of the phosphate content of GPI needs to be corrected by 100 fold. The ratio NH₂ /phosphate of 50:1 therefore becomes phosphate/ NH₂ of 1:2, and 2 moles PO₄/GPI. This is quite similar to Mato et al (Mato et al 1987a) who reported a ratio 3:1 in GPI of rat liver.
Figure 2.6: TLC plates of GPI extracts from total plasma membrane of a normal (A) and a pre-eclamptic (B) placenta. GPI is detectable in the normal but not in the pre-eclamptic sample. The extraction was performed as described in Materials and Methods. Briefly, lipids was first extracted from plasma membrane, apolar lipids were then discarded and finally GPI were isolated by chromatography. Photograph of the plates was taken after iodine staining.
Figure 2.7: TLC of lipid extracted from total plasma membrane of normal placenta, detected by iodine (A) and anisaldehyde (B) staining. Lipids were extracted as described in Materials and Methods. Lanes 1 represent the extract obtained after the first chromatography and lanes 2 represent the final GPI extracts.
Figure 2.8: TLC of GPI extracted from total plasma membrane (lane 1) and microvillous membrane (lane 2). Identical staining is observed in the two lanes. GPI has been extracted as described in Materials and Methods with starting membrane preparations containing 10 mg of protein. The detection of GPI was performed with iodine staining.
Figure 2.9: Determination of amino group content in GPI. The fluorescence induced by the glucosamine standard and the glucosamine standard plus GPI were determined for correction of the matrix effect. After incubation of GPI and known amount of glucosamine in presence of fluorescamine, fluorescence was determined using an excitation wavelength of 380 nm and an emission wavelength of 470 nm.
Figure 2.10: Absorbance of the phosphate standard used for determination of phosphate in GPI and absorbance of the phosphate standard plus GPI. The two lines are parallel showing that there is no matrix effect. Samples and standards were evaporated to dryness; after perchloric acid treatment, ammonium molybdate and ascorbic acid were added and the mixtures were heated. The phosphate content was determined by measuring optic density at 650 nm.
Figure 2.11: Linear dose relationship between phosphate and GPI. Phosphate content was measured in GPI extract as described in Materials and Methods. For this determination, volumes of 5, 10 and 20 µl were found to contain 2.3, 4.4 and 7.9 nmol of phosphate respectively.
Figure 2.12: Phosphate absorbance measurements in known amounts of phosphatidylinositol and inorganic phosphate solutions (From Lance Turtle, University College London).
DISCUSSION

Findings
The work in this chapter describes the extraction of a glycolipid from placenta following a method used to extract the glycolipid GPI precursor molecule of IPGs from human liver. The glycolipid was extracted from total placental membrane as well as the syncytiotrophoblast microvillous membrane. This is the first report of the extraction of free GPI from placental tissue. However, this GPI was not detected in the total plasma membrane or the syncytiotrophoblast microvillous membrane of pre-eclamptic placentae.

GPI was extracted following a method used to extract liver GPI established in our laboratory (Caro et al 1997). As represented in Figure 2.4, the first step of the extraction was to extract lipid from a membrane fraction, the second step isolated polar lipids using the property of silica to bind polar groups after its activation and finally GPI was isolated by further TLC.

The lipid character of the molecule was confirmed by staining with iodine; the glycan part was shown by staining with anisaldehyde and orcinol; the phosphate content was measured and the amino groups were detected using fluorescamine. Phosphate and amino group content may allow quantification of the product. The ratio of phosphate/ amino group obtained is 2:1. It is very close to the one proposed by the group of Mato (Mato et al 1987b, Jones et al 1997) who obtained a ratio of 3:1 with the rat and human liver GPI preparations. This suggests that the glycolipids of placenta and liver differ in their phosphate content. It is also possible that the content of different subtypes of GPI differs in each preparation. Jones et al (Jones et al 1997) demonstrate that their preparation contains at least three different glycolipids which they separated by two dimensional TLC. Our preparation contains at least two glycolipids as seen in Figure 2.6.

The method used to measure the phosphate content of the preparation was very similar to the one used by Jones et al (Jones et al 1997). The standard they used was disodium hydrogen phosphate, we used KH$_2$PO$_4$. The treatment with 70% perchloric acid was 30 min, ours 45 min. After the addition of ammonium molybdate and ascorbic acid, absorbance was determined.
This assay quantifies the amount of inorganic phosphate residue, and in molecules such as GPI and phosphatidylinositol, treatment is necessary to release phosphate. It is the reason why a treatment with perchloric acid is performed before the detection. However, this treatment seems to be inefficient and we had to correct the values obtained with the KH₂PO₄ standard with a standard of PI. Jones et al (Jones et al 1997) did not mention the need for a correction in their report.

*Presence of GPI in microvilli*

GPI has not only been extracted from total plasma membrane but also from microvillous membrane preparations. The location of the IPG precursor GPI in plasma membrane is not surprising as IPG precursor GPI has already been located at the plasma membrane of rat liver (Alvarez et al 1988) and IPGs have been released from placental plasma membrane (Suzuki et al 1984).

In addition, GPI molecules have been located at the surface of parasites (McConville and Ferguson 1993); and in mammalian cells they were found to be principally located in the plasma membranes (Alvarez et al 1988). In polarised epithelial cells of Madine-Darby canine epithelial cells, GPI has not only been located in the apical pole, as is the case for GPI-anchored proteins, but also in the basal plasma membrane (van't Hof et al 1995). As syncytiotrophoblast is also a polarised epithelium, a similar distribution may exist.

The presence of GPI in microvilli supports the results of Chapter 1 (presence of IPGs in microvilli) and the proposed existence of the IPG/GPI signalling system in placenta (as stated in Chapter 1).

*Absence or low level of GPI in pre-eclamptic placenta*

The present finding that GPI has not been detected in pre-eclamptic placentae whereas it is present in normal placentae provides an additional insight into the defect in the IPG/GPI signalling system which may underly pre-eclampsia. Thus the Figure 1.10 can be updated into Figure 2.13 which illustrates the presence and absence (or low level) of GPI in the microvillous membrane of normal and pre-eclamptic placenta respectively.

The absence or an undetectable amount of GPI in pre-eclamptic placenta may be due to a defect of the biosynthesis of the glycolipid. The presence of IPG activity in pre-
eclamptic placenta suggests however that the precursor has been synthesised. Another explanation for the absence or an undetectable amount of GPI in pre-eclamptic placenta might be a complete catabolism of the molecule resulting in degradation of the total pool of GPI. A higher hydrolysis of GPI is consistent with the higher levels of IPGs reported in the placenta (Chapter 1 and Kunjara et al 2000a). In this regard, GPI-PLD, the enzyme possibly responsible for GPI hydrolysis in normal and pre-eclamptic placentae is the subject of investigation in the following chapter.

It is interesting to note that the distribution in the microvillous membrane of alkaline phosphatase is impaired in the pre-eclamptic placenta (Jones and Fox 1976, Jones and Fox 1980). Thus both GPI and alkaline phosphatase, a GPI-anchored protein, are present in lower amounts in the brush border membrane of pre-eclamptic placentae than in the plasma membrane of normal placentae. Both suggestions proposed to explain the deficiency of GPI in pre-eclampsia, namely the defect in GPI biosynthesis and the increased GPI degradation, may also be applied to alkaline phosphatase. In addition, circulating placental alkaline phosphatase is higher in pre-eclampsia than in normal gestation (Benster 1970, Adeniyi and Olatunbosun 1984) and IPG P-type activity is higher in urine of pre-eclamptic women compared to matched pregnant women (the source of these IPGs was believed to be the placenta) (Kunjara et al 2000a). This implies a higher release into the circulation of these molecules from the placenta. Since both GPI and the GPI-anchored form of alkaline phosphatase share the ability to be cleaved by GPI-specific phospholipase, it can be conceived that in pre-eclampsia the explanation for decreased amounts of these molecules in the brush border membrane, results in both cases not from a defect of GPI biosynthesis, but from increased hydrolysis. (The hydrolysis of GPI results in water-soluble IPGs and the hydrolysis of GPI-anchored alkaline phosphatase results in a lower molecular weight form of alkaline phosphatase devoid of the lipid moiety).

An other explanation of low levels of alkaline phosphatase and undetectable amount of GPI in pre-eclamptic placenta is the increased loss of microvilli.

Glycolipid composition of microvilli
The microvillous membrane of normal and pre-eclamptic placenta differs. In pre-eclampsia, microvilli are reduced in number, or disturbed with thickened bulbous tips.
giving them an abnormal club-like shape (Jones and Fox 1980). The shape of caveolae has been reported to be dependent on the cholesterol content (Anderson 1998). A different lipidic composition and more particularly the cholesterol content may differ in normal and pre-eclamptic placenta and would explain the difference in microvilli shape. As GPI has not been detected in microvilli preparations of pre-eclamptic placentae, it is also possible that this lipid plays a role in the shape of the microvilli.

Furthermore, the disruption of caveolar integrity has been reported to eliminate insulin signalling (Stralfors 1997). The change in microvilli shape seen in pre-eclampsia may therefore reflect an altered lipid content which could result in a change in caveolar integrity. This could result in altered IPG/GPI signalling which we believe may be the basis of this disorder.

Conclusion

A glycolipid, thought to be an IPG precursor molecule has been extracted from total plasma membrane and microvilli preparations of normal placenta, but has not been detected in pre-eclamptic samples. It is possible that abnormal degradation of GPI is due to an overstimulation of a specific GPI-phospholipase. In the next chapter the phospholipase present in the microvilli of normal and pre-eclamptic placenta has been identified and its expression has been investigated.
Normal Pre-eclampsia

Figure 2.13: Hypothetical model of IPG release in the microvillous membrane of normal and pre-eclamptic placenta. GPI has been detected in microvillous membrane of normal placentae but not in microvillous membrane of pre-eclamptic placentae. IPG maybe released from microvillous membrane after stimulation of a GPI-specific phospholipase, such as GPI-PLD, which would hydrolyse GPI to generate IPGs. A complete degradation of the pool of GPI by an abnormal stimulation of GPI-PLD may explain the higher level of released IPG. Abbreviations: GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; IPG, inositolphosphoglycan; STB, syncytiotrophoblast.
Chapter 3

GPI-PLD in Placenta
INTRODUCTION

*What is GPI-PLD?*
Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD, also known as GPLD and PIG-PLD) (EC 3.1.4.50) belongs to the large family of phospholipases (EC 3.1-), enzymes which hydrolyse phospholipids. The hydrolysed bond of the substrate determines the nature of the phospholipase. As indicated in Figure 3.1, a phospholipase D (PLD) cuts between the phosphate and polar head group, therefore producing phosphatidic acid (PA). The bonds hydrolysed by other types of phospholipases are also indicated in the figure.

Phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine are PLD substrates but not GPI-PLD substrates. To be hydrolysed by GPI-PLD, the substrate requires a GPI motif containing a glycan core attached to a phosphatidyl inositol. The glycan core can be very simple since a GPI biosynthetic precursor consisting of Man1-GlcN-phosphatidylinositol has been found to be cleavable by GPI-PLD (Masterson *et al* 1990). The motif recognised by GPI-PLD is a conserved portion of GPI-anchored proteins and free GPI molecules. This latter molecule was considered in the previous chapter.

![Figure 3.1: Scheme indicating the site of cleavage of phospholipase D on phospholipids molecules. The bonds hydrolysed by other types of phospholipases, which are named according to their specificities, are also indicated.](image)

---

**Figure 3.1:** Scheme indicating the site of cleavage of phospholipase D on phospholipids molecules. The bonds hydrolysed by other types of phospholipases, which are named according to their specificities, are also indicated.
**Discovery of GPI-PLD activity**

GPI-PLD activity was discovered simultaneously by three different groups in the late eighties in human serum and plasma (Davitz et al 1987, Cardoso de Almeida et al 1988, Low and Prasad 1988) and human placenta (Malik and Low 1986).

In 1987, Davitz et al reported a GPI-PLD activity in human serum which cleaved the hydrophobic form of the GPI-anchored protein decay accelerating factor (DAF) into a soluble one. The lipase activity was not effective on phosphatidylethanolamine, phosphatidylcholine, or phosphatidylinositol, but was effective on the GPI-anchored protein, membrane form variant surface glycoprotein (mfVSG). The activity was found to be Ca\(^{2+}\) dependent, heat labile, and not affected by the inhibitor of serine proteases PMSF (Davitz et al 1987).

In 1986, Malik and Low proposed that a GPI-PLD, and not a PLC activity was responsible for the cleavage of the high molecular weight form of alkaline phosphatase into the lower molecular weight form in the placenta (Malik and Low 1986). Later, the same group reported high GPI-PLD activity in plasma and/or serum of different mammals (human, rat, rabbit, fetal bovine, chicken and horse) (Low and Prasad 1988). The activity was detected when the substrates used were GPI-anchored proteins, i.e. alkaline phosphatase, 5'-nucleotidase and mfVSG, and could not be detected using phosphatidylcholine or phosphatidylinositol, confirming the GPI specificity of the phospholipase. The lipid product identified was phosphatic acid (PA) confirming the D nature of the lipase. 1-10 phenanthroline, a chelator of transition metal ions inhibited the activity, EGTA and the thiol-blocking agent p-hydroxymercuriphenylsulphonic acid also inhibited the activity but to a lesser extent. A third group, (Cardoso de Almeida et al 1988) also reported a human serum GPI-phospholipase activity which was not C type. The demonstration was based on the recognition of a cryptic carbohydrate (CRD) epitope which is only present after GPI-PLC cleavage of GPI-anchored proteins. The GPI-PLC cleavage exposes the CRD epitope present on the C-terminus of many GPI-anchored proteins, which is composed in part of a terminal inositol-phosphate group. GPI-PLD cleavage eliminates the terminal phosphate on the inositol thus destroying the CRD epitope.

**Purification of the protein**

Soon after the discovery of the enzymatic activity, the enzyme was purified by two of the previously mentioned groups (Davitz et al 1989, Huang et al 1990). In 1989
(Davitz et al 1989), GPI-PLD was purified to homogeneity from human plasma. The enzyme was identified as a single polypeptide chain of 110kDa. (5% of the total enzymatic activity was recovered after a 4,500 fold purification.) GPI-PLD was identified as a glycoprotein since it bound to a wheat germ lectin column, and the enzyme had a pH range of activation from 4.0 to 8.5. The other group (Huang et al 1990) purified GPI-PLD from bovine serum and found a protein of similar molecular weight (100 kDa) with an isoelectric point of 5.6. The concentration of GPI-PLD in serum was estimated at 7 μg/ml. Whereas amino terminal sequence analysis of the intact 100 kDa protein revealed no strong homology to that of any other known proteins, two of the twelve tryptic peptides shared sequence homologies with each other and with the metal ion binding domains of members of the integrin family. Later, a 47kDa form of GPI-PLD was also reported in bovine serum (Hoener et al 1994). This 47 kDa active form of GPI-PLD was the N-terminus of the 115 kDa form and was probably a product of proteolytic cleavage by trypsin. It was also shown that bovine serum GPI-PLD can be cleaved into three tryptic fragments of 33, 39 and 47 kDa. The PLD activity was associated with the 39 kDa N-terminal region of GPI-PLD (Heller et al 1994). Whereas the N-terminal region contains the active site (Heller et al 1994), it has also been shown that the C-terminus was essential for catalytic activity (Stadelmann et al 1997). GPI-PLD activity was not detected in cells transfected with GPI-PLD in which 6 to 8 C-terminal amino acids from a total of 816 amino acids were deleted. The loss of activity was probably due to misfolding implying thus that the C-terminal part of the protein was involved in maintaining the structure of GPI-PLD. 

**GPI-PLD mRNA expression**

The first complementary DNA (cDNA) clone of GPI-PLD was obtained in 1991 from a bovine liver library (Scallon et al 1991). It was isolated on the basis of the amino acid sequence of eight of the tryptic fragments of GPI-PLD generated by the group which purified the protein from bovine serum (Huang et al 1990). The cDNA encoded a mature protein of 817 amino acids with a predicted molecular weight of 90.2 kDa with eight potential sites for N-linked glycosylation.
One GPI-PLD cDNA has also been identified in the mouse (LeBoeuf et al 1998) and rats (Schofield and Rademacher 2000) and two different human cDNAs have been reported from liver (L11701) and pancreatic islets (L11702) (Tsang et al 1992). Our group has only found one of these two human GPI-PLD cDNAs and has shown that only one GPI-PLD gene exists in the human genome. The structure of the gene, which covers approximately 100 kb on chromosome 6p22 has been determined (Schofield and Rademacher 2000). The study showed that the liver is the site of the most abundant GPI-PLD gene expression. It is noteworthy that this does not mean that hepatocytes are the cells expressing GPI-PLD, it might be other endogenous cells of the liver as liver cell lines do not express GPI-PLD (Personal communication Dr JN Schofield, University College London). However, cell-lines are not normal cells, and may show changes in cell function.

**Regulation of GPI-PLD activity**

Regulation of GPI-PLD activity is important since the high concentration of the enzyme in serum would be sufficient to remove most of the GPI-anchored proteins on the surface of a cell in less than one minute (Low 1990).

GPI-PLD is inhibited by the divalent cation chelators EDTA and EGTA (Malik and Low 1986, Davitz et al 1987, Low and Prasad 1988, Davitz et al 1989, Hoener et al 1990, Huang et al 1990). Since the inhibitory effect of EDTA and EGTA can be blocked by addition of Ca$^{2+}$, it has been proposed that GPI-PLD is Ca$^{2+}$ regulated (Davitz et al 1987, Hoener et al 1990). Furthermore, GPI-PLD has been found to contain four calcium binding sites, E-F hand-like domains, that were also found in integrins (Huang et al 1990, Scallon et al 1991, Heller et al 1994). However Ca$^{2+}$ was found relatively ineffective at blocking the inhibitory effect of EGTA compared with Zn$^{2+}$ (Li et al 1994). In addition Zn$^{2+}$, but not Ca$^{2+}$, was also able to block the inhibitory effect of 1,10-phenanthroline on GPI-PLD. Thus the inhibitory effect of GPI-PLD induced by EDTA, EGTA and 1,10-phenanthroline was likely due to other metal ions. The involvement of Zn$^{2+}$ rather than Ca$^{2+}$ in GPI-PLD activity has been proposed (Li et al 1994). The metal analysis of GPI-PLD revealed 5 atoms of Ca$^{2+}$ and 10 of Zn$^{2+}$ per molecule of GPI-PLD (Li et al 1994).

Interestingly enough, purified GPI-PLD is not active upon substrate incorporated in intact cell membrane (Low and Prasad 1988) unless detergent (Low 1990, Low and
Huang 1991) or a cholesterol-binding agent (Bergman and Carlsson 1994) is added to the reaction mixture, thus suggesting that the lipidic environment modulates GPI-PLD activity. Furthermore, phosphatidic acid, lysophosphatidic acid and lipid A are three lipids which inhibit GPI-PLD activity (Low and Huang 1993). Similarly, GPI-PLD activity in large unilamellar vesicles varies depending on the lipidic composition. For example, GPI-PLD hydrolyses 81% of GPI in vesicles made of GPI/phosphatidylcholine/phosphatidylethanolamine with a ratio of 1:1:2, whereas in the presence of phosphatidylethanolamine and sphingomyelin (GPI/phosphatidylethanolamine/sphingomyelin with a ratio of 1:2:1), GPI-PLD activity drops to 14% (Villar et al 1998).

The role of cholesterol also seems important. It has been reported that the presence of a cholesterol-sequestering substance (saponin) allows GPI-PLD hydrolysis of membrane-attached Thy-1 (Bergman and Carlsson 1994). Furthermore, cholesterol is a major component of caveolae, microdomains where GPI-anchored protein are clustered (Anderson 1998), and its depletion, induced by addition of cholesterol-binding substances (saponin) or by inhibitors of sterol synthesis, causes a dispersal of GPI-anchored proteins in the membrane (Rothberg et al 1990, Cemeus et al 1993). It has therefore been suggested that the susceptibility of GPI-anchored proteins to cleavage by GPI-PLD can be determined by the clustering of GPI-anchored proteins, which is controlled by lipid-lipid interactions between the fatty alkyl/acyl chains of the GPI anchor and the surrounding lipids in the bilayer (Bergman and Carlsson 1994).

Phosphorylation of GPI-PLD also modulates its activity. Recently, GPI-PLD has been shown to be phosphorylated by cAMP-dependent protein kinase A in vitro. The phosphorylated form exhibits a reduced activity, which is restored by alkaline phosphatase treatment (Civenni et al 1999). Analysis of the amino acid sequence also reveals potential phosphorylation sites for CKII and protein kinase C (motif search programme, HGMP). Thus regulation of GPI-PLD by phosphorylation maybe a significant factor in controlling this enzyme activity.

It is also interesting to note that bovine serum GPI-PLD is still active after proteolytic cleavage by trypsin, which generates 3 fragments (2 of about 40 kDa, and a carboxy terminal fragment of 30 kDa) (Li et al 1994). There is little change in
activity at low trypsin concentrations (0.04 μg/ml) but a 3-4 fold increase in GPI-PLD activity occurs at higher concentrations (40μg/ml). This increase in activity is also obtained when GPI-PLD is treated with other proteases.

Functional role

When GPI-PLD cleaves a GPI-anchored protein, it releases a soluble protein and PA; when it cleaves free GPI, it releases IPGs and PA. Thus GPI-PLD could be involved in the release of proteins from the membrane as well as generating the biologically active compounds, PA and IPGs, which are involved in signal transduction processes.

The role of GPI-PLD in releasing GPI-anchored proteins from the cell surface in vivo was proposed when the enzyme was first discovered (Low and Prasad 1988). The hydrolysis, inducing the release of the protein from the cell membrane, may therefore regulate the availability of the protein in the extra-cellular medium and more generally in the circulation. In this regard, a role for GPI-PLD has been proposed in hematopoiesis (Brunner et al 1994). Basic fibroblast growth factor (bFGF) is a potent stimulator of stem cell growth and hematopoiesis. bFGF binds to the GPI-anchored heparan sulfate proteoglycan on the surface of bone marrow stromal cells forming a complex which can be released by GPI-PLD hydrolysis and which mediates the biological action of bFGF (Brunner et al 1994). The release of a soluble form of Thy-1 by GPI-PLD in human cerebrospinal fluid has also been proposed (Bergman and Carlsson 1994), as the protein was found to contain the glycan moiety of GPI, but lacked the PA part (Almqvist and Carlsson 1988).

The release of soluble alkaline phosphatase in the circulation has also been shown to be associated with GPI-PLD (Malik and Low 1986, Deng et al 1996).

Apart from the release of membrane-anchored proteins, GPI-PLD cleavage has also been postulated to have an important role in intracellular signalling, through the generation of PA (Davitz et al 1987) and IPGs (Jones et al 1997). PA and its lysoderivatives exert hormone-like and growth factor-like activities such as Ca$$^{2+}$$ mobilisation, stimulation of DNA synthesis and cell division (Moolenaar et al 1986, Moolenaar et al 1992, Jalink et al 1990, Jalink et al 1994).

It has been postulated that IPGs could be released from hydrolysis of GPI by GPI-PLD (Jones et al 1997, Rademacher et al 1994). Therefore GPI-PLD can be
implicated in the numerous functional roles of IPG, including stimulation and inhibition of biological enzymes and cellular proliferation (Varela-Nieto et al 1996). The enzyme may consequently be involved in placental steroidogenesis (Nestler et al 1991) or gluconeogenesis in the pre-eclamptic placenta (Kunjara et al 2000a).

**Tissue expression**

GPI-PLD is present in high amounts in the circulation. Its concentration in human plasma has been estimated at 5-10 µg/ml (Davitz et al 1989, Hoener and Brodbeck 1992). A portion of bovine serum GPI-PLD combines with high density lipoprotein lipase (Hoener and Brodbeck 1992) and interacts with apolipoprotein AI (Deeg 1994). As GPI-PLD binds lipids and is associated with apolipoprotein AI in serum, the enzyme can be defined as an apolipoprotein (Deeg 1994).

The presence of a bovine serum 47 kDa GPI-PLD active form with identical N terminus to the full-size enzyme, (corresponding probably to a tryptic digest) has also been reported in bovine serum (Hoener et al 1994).

The high amount of GPI-PLD in the circulation raises the question of its site of synthesis. GPI-PLD activity, immunostaining or both activity and immunostaining has been reported in several tissues such as placenta (Malik and Low 1986), brain (Hoener et al 1990), pancreas (Metz et al 1991, Deeg and Verchere 1997), liver (Scallon et al 1991, Heller et al 1992), and in different physiological fluids, such as milk and cerebrospinal fluid (Hoener and Brodbeck 1992). The cells where GPI-PLD has been detected are motor and sensory neurons (Seso and Low 1991), keratinocytes (Xie et al 1993), mast cells of liver, adrenal gland and lung (Metz et al 1992, Stadelmann et al 1993), and several cell lines, including mouse-derived insulinoma, (Deeg and Verchere 1997), myeloid and HeLa cell lines (Xie and Low 1994).

The GPI-PLD transcript however has only been detected in liver (Scallon et al 1991), in both α and β cells of the pancreatic islets of Langherhans (Metz et al 1991), in mast cells (Metz et al 1992, Stadelmann et al 1993), and in HeLa, myeloid- (Xie and Low 1994) and pancreatic-derived cell lines (Deeg and Verchere 1997). The presence of mRNA in liver was attributed to endogenous mast cells (Stadelmann et al 1993). Some groups postulated that serum GPI-PLD is secreted by the pancreas.
(Deeg and Verchere 1997), others postulated myeloid cells as a potential contributor to the circulating GPI-PLD pool (Xie and Low 1994).

Taken together, the high concentration of GPI-PLD in serum and the lack of detection of synthesis of GPI-PLD in the majority of body tissues, where the protein is detected either by its activity or by immunostaining, suggests that the majority of cells may fulfill their requirement for GPI-PLD by taking the enzyme up from serum. This phenomenon occurs in mouse neuroblastoma cells in vitro (Hari et al 1997).

**Cellular localisation**

The cellular localisation of GPI-PLD has been investigated in myeloid cells (Xie and Low 1994). All human myeloid cell lines tested showed a similar pattern of staining with an antibody reactive with bovine serum GPI-PLD. There were bright dots in the perinuclear region and around the surface of the cells. The punctate immunostaining suggested a concentration of GPI-PLD in a limited intracellular location, presumably secretory vesicles. Further experiments of permeabilisation of cells and fractionation by sonication suggested that intracellular GPI-PLD was localised mainly in vesicles with the remainder in the endoplasmic reticulum and Golgi.

The demonstration of a post-Golgi compartment location was performed using brefeldin A, which is known to block the transport of protein across the Golgi (Lippincott-Schwartz et al 1990). In this experiment, GPI-PLD release was weakly inhibited by brefeldin A.

The same group had previously investigated the localisation of GPI-PLD in keratinocytes (Xie et al 1993). The immunostaining experiments and sonication fractionation experiments showed different patterns suggesting different intracellular localisation (Xie and Low 1994).

In rat liver cells, GPI-PLD has been found mainly localised in the lysosomal compartment (Hari et al 1996). The presence of GPI-PLD in this acid fraction is consistent with its broad pH spectrum of activity (Davitz et al 1989) and its activity following proteolytic cleavage (Heller et al 1994).

Similarly, GPI-PLD has been found in an acidic intracellular compartment in neuroblastoma cells. The authors proposed an uptake of GPI-PLD from the medium and transit in an acidic intracellular compartment (probably lysosome) in which GPI-PLD was fragmented into smaller active GPI-PLD forms (Hari et al 1997).
Thus, GPI-PLD has been located at the cell surface and internally in post Golgi vesicles, which are presumed to be lysosomes.

*Reasons for investigating GPI-PLD in the placenta*

The presence of GPI-PLD activity in the human placenta has already been proposed (Malik and Low 1986). The GPI-PLD activity was proposed to cleave the hydrophobic, high molecular weight form of alkaline phosphatase into a hydrophylic, low molecular weight form. However, the location in the placenta remains to be determined. Trophoblast, the major component of placenta may contain the enzyme. More precisely, the presence of the enzyme in the microvillous membrane can be expected since its substrates, GPI-anchored proteins and free-GPI are known to be localised in this part of the membrane (Chapter 2). GPI-PLD would be another component of the machinery of the IPG/GPI signalling system. It would release IPGs from GPI.

In addition, a defect of GPI-PLD activity in pre-eclampsia may explain the high levels of IPGs and the absence of detectable GPI in the pre-eclamptic placenta. A defect of GPI-PLD activity in the pre-eclamptic placenta is also consistent with an explanation for the lower level of alkaline phosphatase activity in pre-eclamptic placentae and more specifically in the microvillous membrane of pre-eclamptic placentae. GPI-PLD would hydrolyse the GPI anchored-form of alkaline phosphatase releasing the soluble form into the circulation, which would therefore be in higher concentration in pre-eclampsia than in normal pregnancy.

In addition to investigating the placental location and activity levels of GPI-PLD in normal and pre-eclamptic placenta, other issues concerning this enzyme remain to be resolved. The first concerns its size. GPI-PLD isolated from blood has a molecular weight of 100-120 kDa, although a 47 kDa active form is also present in the bovine serum. The size of the placental form of GPI-PLD remains to be determined.

The second issue is the origin of placental GPI-PLD. The presence of GPI-PLD in the placenta may be due to either *de novo* synthesis by the placenta or by direct uptake from the maternal blood where GPI-PLD is present in high concentration. These issues have been addressed in this chapter.
Aim of the chapter

The aim of the work described in this chapter has been to investigate the presence of GPI-PLD in normal and pre-eclamptic placenta and more specifically in the syncytial brush border membrane facing the maternal blood. The protein was physically detected by immunocytodetection and Western-blotting and its activity measured. Expression of the GPI-PLD gene (GPLDI) was investigated by RT-PCR.
MATERIALS & METHODS

1- Immunohistochemistry

The detection and localisation of GPI-PLD in human placental villi was determined by immunohistochemistry using an anti-bovine GPI-PLD polyclonal antibody following a slightly modified method described in Chapter 1 for the detection of IPG.

Materials

Cryosections were from the same placentae as those used for immunostaining of IPG (Chapter 1). Polyclonal anti-GPI-PLD was a gift from Dr Gilfillan from Hoffman-La Roche Inc (Nutley, UK), preimmune serum was a gift from Dr P Williams (University College London), swine anti-rabbit IgG conjugated to horseradish peroxidase (HRP) was obtained from Dako corp (Slough, England) and bovine serum albumin (BSA) was purchased from Sigma Aldrich Ltd (Poole, England).

Method

Prior to labelling with antibodies, the sections were allowed to equilibrate at room temperature, and non-specific absorption was blocked with 5%BSA in PBS for 60 min at room temperature. Immunostaining was via indirect immunohistochemistry using a rabbit polyclonal IgG antibody (anti bovine GPI-PLD). This was incubated at 10μg/ml in PBS containing 0.5% BSA for 60 min at room temperature in a humidified atmosphere followed by three brief washes of PBS. Controls for the antibody were conducted using rabbit preimmune serum under the same conditions. The secondary antibody detects the presence of the primary antibody and hence the original antigen of interest, GPI-PLD. The secondary antibody was a swine anti-rabbit IgG conjugated to horseradish peroxidase (HRP) used at a dilution of 1/100. It was placed onto the tissue sections and incubated for another 60 min at room temperature in a humidified atmosphere and sections were then washed and incubated for 10 min with 3,3’-diaminobenzidine tetrahydrochloride solution to develop the brown dye corresponding to the presence of HRP; they were stained with Haematoxylin and mounted in Styrolyte mounting medium. Specimens were then examined through immersion oil. Photographs were taken.
2 - Western-blot

Materials

Protogel, Protogel buffer, Protogel stacking buffer, Tris glycine SDS buffer were obtained from Flowgen (Ashby de la Zouch, UK). N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate were purchased from Sigma Aldrich Ltd (Poole, England). Recombinant molecular weight markers, nitrocellulose membrane, ECL+, and autoradiography films were obtained from Amersham Life Science (Uppsala, Sweden). Powder milk was Marvel from a grocery. Tween 20 was obtained from BDH Ltd (Poole, England). Antibodies were the same as those used for immunohistochemistry (page 127).

Method

GPI-PLD detection, in homogenates of placenta and microvilli preparations from normal and pre-eclamptic placentae, was determined by immunoblotting. Placental villi tissue was homogenised in a suspension buffer (0.1M NaCl, 0.01M Tris HCl (pH 7.6), 0.001M EDTA (pH 8.0), 1 µg/ml aprotinin and 100 µg/ml phenylmethylsulfonyl fluoride) and microvilli preparations were diluted in the same buffer so that each sample contained 0.3 mg of protein. They were boiled for 10 min in an equal volume of SDS gel loading buffer consisting of 50 mM Tris HCl, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol. A volume of 40 µl of each sample was loaded onto a 6% acrylamide stacking gel and run under 100 volt. When the dye front had moved into the underlying 8% acrylamide resolving gel, the voltage was increased to 200 volts (The electrophoresis buffer contains 0.025 M Tris, 0.192 M glycine, 0.1% SDS). After separation by SDS/PAGE, proteins were transferred overnight to a nitrocellulose membrane under a current of 25 mA (the transfer buffer contained 39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol). Before probing the membrane, non specific binding sites were blocked by immersing the membrane in a solution of 5 per cent milk in phosphate buffer saline containing 0.1 per cent Tween (PBS-T) for one hour at room temperature using an orbital shaker. After washing with PBS-T, the membrane was incubated for one hour with the polyclonal rabbit anti-bovine GPI-PLD antibody diluted at 0.1 µg/ml in the milk/PBS-T solution, washed again and incubated with the secondary antibody,
horseradish peroxidase-conjugated swine anti-rabbit antibody at 0.1g/ml (dilution 1:2000, stock solution 0.24g/l). After washing, the presence of peroxidase was detected by chemiluminescence using the ECL detection reagents and exposure to autoradiography film for 1-10 min to visualise the GPI-PLD bands.

3- GPI-PLD activity assay using mfVSG

Materials

$[^{3}H]$mfVSG was a gift from Dr K Puan, University College London. Silica gel G60 thin-layer chromatography (TLC) plates were purchased from BDH Ltd (Poole, England). Nonidet P-40 (NP-40) was obtained from ICN Biomedicals Inc (Aurora, Ohio, USA). Sodium fluoride and sodium orthovanadate were obtained from Sigma Aldrich Ltd (Poole, England). Ultima Gold was obtained from Packard Bioscience (Groningen, The Netherlands). Bovine GPI-PLD was obtained from Boehringer (Ingelheim, Germany). PI-specific phospholipase C from B. thuringiensis was obtained from Oxford GlycoSciences Ltd (Abingdon, UK). En3Hance Spray was obtained from NEN Life Science Products (Boston, USA) and X-ray films were purchased from Fuji (Hemel Hempstead, UK).

a) GPI-degradation assay

The GPI-degrading activity of microvilli preparations was monitored using mfVSG as substrate as described by (Huang et al 1990) with some modifications. Microvillous membrane preparations, containing a known amount of protein, were pelleted and suspended in 50 µl of buffer A (150 mM NaCl, 10 mM Hepes pH 7.0, 0.1 % NaN3) with 0.5% Nonidet P40. After a 20 min incubation on ice, the solution was centrifuged for 5 min at 13,000 rpm and 10 µl of the extract supernatant (containing 0.12 to 0.50 mg of protein) was added to 90 µl of buffer A (100 µl of buffer A for negative control sample), to which 100 µl of substrate mixture containing 6 µg of $[^{3}H]$mfVSG and 40 mM Tris/maleate (pH 7.0), 0.1% NP-40 was added. The reaction was incubated at 37°C for 15, 30 or 60 min and then stopped by the addition of 0.5 ml of 1M NH4OH-saturated butanol. After vortex mixing, phase separation was achieved by centrifugation for 3 min at 13 000 rpm. Then 0.3 ml of
the upper organic phase was removed, mixed with 10 ml of Ultima Gold and analysed for radioactivity by liquid-scintillation counting. One unit of GPI-PLD activity is arbitrarily defined as the amount of enzyme required to hydrolyse 1% of \[^3H\]mfVSG in 1 min.

b) Identification of products of GPI-anchor converting activity by TLC analysis of mfVSG

To identify the lipidic products of mfVSG degradation, the reaction was performed as described above with slight modifications. The degradation was induced either by placental membrane extract (containing 0.05 mg of protein) with or without phosphatase inhibitors (50 mM sodium fluoride and 2.6 mM sodium orthovanadate), or by 0.5 unit of bovine GPI-PLD or 0.1 unit of bacterial PI-PLC. The incubation time of the reaction was lengthened to 3 h. After phase separation 300 μl of the organic phase was evaporated using a speed-Vac concentrator and the lipids were resuspended in 20 μl of chloroform/methanol (2:1 v/v) before spotting onto a TLC glass plate. The plates were developed in chloroform/methanol/0.25% KCl (55:45:10 v/v/v), air dried, sprayed with En3Hance, and radioactivity on the TLC plates was visualised by autoradiography using Fuji RX X-ray films after two weeks exposure.

4- GPI-PLD mRNA investigation

In order to study the expression of GPI-PLD in the placenta, RT-PCR was performed on (1) cDNA synthesised from total RNA extracted from our placental samples, (2) two placental cDNA libraries, and (3) cDNA synthesised from total RNA extracted from BeWo cells.

Materials

Placental tissue consisted of pieces of frozen placentae from UCL Hospital. They were from the same source as those used for GPI extraction (Chapter 2). Taq DNA Polymerase and RNase inhibitor were from Promega Corp (Madison, USA). RNAzol™ B kit was from Biogenesis Ltd (Poole, England). PdN6 random hexomeres were from Pharmacia Biotech (Uppsala, Sweden). dGTP, dATP, dTTP, dCTP were from MBI Fermentas (S' Leon-Rot, Germany). Superscript RT, DTT and
1Kb marker were from Gibco BRL - Life Technologies Ltd (Paisley, UK). Primers were constructed by Cruachem Ltd (Glasgow, UK). The full-length GPI-PLD cDNA clone was a gift from Dr JN Schofield and the human skeletal muscle cDNA library was from Clontech (Palo Alto, USA). Agarose was from Boehringer (Ingelheim, Germany). Ethidium bromide, bromophenol blue, xylene cyanol ff and glycerol were from Sigma Aldrich Ltd (Poole, England).

**Methods**

**a) RNA extraction**

Human placental tissue was snap-frozen in liquid nitrogen within 30 min of collection and stored at -80°C. Total RNA was extracted using the RNAzol™ B reagent. The method is based on the property of RNAzol to promote formation of complexes of RNA with guanidium and water molecules, and abolishes hydrophilic interactions of DNA and proteins. Small pieces of placenta were reduced into powder using a pestle and mortar and about 100 mg placental powder was homogenized in 2 ml RNAzol™ B in a homogenizer. RNA was extracted by adding 0.2 ml chloroform per 2 ml of homogenate, shaking vigorously for 15 seconds and leaving on ice for 15 min. The suspension was then centrifuged at 12 000 g (4°C) for 15 min, and the upper aqueous phase containing the RNA was transferred to a fresh tube where an equal volume of isopropanol was added. After 1 night at -20°C, samples were centrifuged for 15 min at 12 000 g (4°C). The RNA precipitate, forming a white pellet at the bottom of the tube was resuspended in diethylpyrocarbonate (DEPC)-treated water (20μl). The amount of RNA was determined by spectrophotometric analysis at a wavelength of 260 nm where 1 OD$_{260}$ unit of absorbance = 40 μg/ml RNA. The yield was approximatively 0.7 μg RNA/mg placenta.

**b) cDNA synthesis**

Total RNA was reverse transcribed to cDNA by incubating 5 μg RNA in 50 mM Tris-HCl (pH 8.3) containing 1 μg pdN6 (random hexomeres), 2 μl of 20 mM dNTPs, 40 U Rnase inhibitor, 3.5 μl of 0.1 M DTT. RNA was first denatured by incubation at 65°C for 5 min. 1.5 μl of Superscript reverse transcriptase, provided in
20 mM Tris-HCl (pH 7.5) buffer containing 100 mM NaCl, 0.1 mM EDTA, 1mM DTT, 0.01% (v/v) NP-40 and 50% (v/v) glycerol, was then added, and incubated at 42°C for 1h30. The final volume of the reaction was 35 µl. Each sample had a negative control in which RNA but no reverse transcriptase enzyme was added. Samples were then stored at -20°C.

c) RT-PCR

In order to check the integrity of the RNA and cDNA synthesis reaction, a β-actin RT-PCR was performed on reverse transcribed samples. Oligonucleotides primers were constructed from the nucleotide sequences found in the GenBank Database. The sequence of the β-actin primers was 5’ ATGGATGATGATATCGCCGC 3’(sense) and 5’ ATCTTCTCGCGGTTGGCCTT 3’ (antisense), corresponding to bases 1093-1580, giving an amplification product of 353 bp in cDNA. A 488 bp product would be obtained if genomic DNA was present as the primers were designated to integrate a 135 bp intron (Nakajima-Iijima et al 1985). The amplification profile consisted of a denaturing step at 94°C for 4 min after 2 min at 40°C (requirement of heated lid PCR machines), followed by 30 cycles of annealing (52.3°C for 30 sec), elongation (72°C for 30 sec) and denaturation (94°C for 25 sec), with a final extension period at 72°C for 5 min.

The sequence of the GPI-PLD primers was 5’ TTCTTGGAGGACTGGATGA TATGGC 3’(P6 sense) and 5’ TGGAGCCACCTATGACATTGTCC 3’ (P13 antisense), corresponding to bases 740-1092, giving an amplification product of 313 bp in cDNA (Tsang et al 1992). A 30 cycle PCR was prepared as above except that an annealing temperature of 63.0°C was used. (Note: the annealing temperature has been calculated using the following formula: Tm = 69.3 + 0.41 × (%GC) – 650/l, Ta = Tm – 5, Tm is the melting temperature, Ta is the annealing temperature, l is the length of the primers in base pairs, %GC represents the percentage of G and C bases.

For each RT-PCR, 5 µl of the cDNA synthesis reaction was incubated in 50 µl PCR reaction, consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton X-100, containing 100 µM of each dNTP, 1.5 mM MgCl2, 0.5 pmole/µl of a sense and antisense oligonucleotide and 1µl of Taq polymerase (Thermus aquaticus DNA polymerase).
polymerase – Taq polymerase is stored in a 20 mM Tris-HCl buffer containing 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% NP-40.)

As a positive control in the GPI-PLD PCR, 1 μl of diluted full-length GPI-PLD cDNA clone isolated from a human liver cDNA library was added in place of cDNA from placentae samples. As a positive control in the β-actin PCR, 2 μl of a human skeletal muscle cDNA library were added in place of cDNA from placentae samples. For the negative control no DNA was added.

Two microliters of loading buffer (0.25% of bromophenol blue, 0.25% xylene cyanol ff and 30% glycerol) were added to an aliquot of 7 μl of PCR reaction. The samples were then submitted to electrophoresis (70V for 60 min) in a 1% agarose gel prepared in TBE containing ethidium bromide (1μl for 50 ml). Ethidium bromide binds to DNA and fluoresces under UV light and thus enables visualisation of DNA in the gel. Photographs of the gel were taken under UV.
RESULTS

Evidence of GPI-PLD protein and GPI-PLD activity in the placenta, but no detectable GPI-PLD mRNA

Immunocytochemistry using a polyclonal antibody reactive with serum bovine GPI-PLD showed that the enzyme is localised in the human placental villi (Figure 3.2). Positive immunoreactivity was present in both trophoblast and internal stroma tissue, and more precisely seems to be localised in the plasma membrane and in the border of placental vessels. The staining was not greater in sections of pre-eclamptic tissue compare to those of normal placenta (n = 3).

The same antibody was used to detect GPI-PLD in a placental homogenate and microvilli preparations and to determine the size of the recognised protein by Western-blotting. Figure 3.3 demonstrates that the antibody binds to a protein in both the homogenates of whole placenta (lane 1) and placental microvilli (lanes 2-6). As shown, a 50 kDa protein has been detected in both a placental homogenate (n=1) and microvilli preparations of both normal (lanes 1-4, 6) and pre-eclamptic placentae (lane 5) (n=5, 3 normal and 2 pre-eclamptic). A fainter band of 100 kDa has also been detected in some but not all microvilli preparations of both normal and pre-eclamptic placentae (lanes 5 and 6). It should be noted that a 100 kDa protein is recognised in bovine serum by Western-blotting using the same polyclonal antibody (data not shown, personal communication from Helen Whitby).

\[^{3}H\]mfVSG was degraded by the microvilli preparation of both normal and pre-eclamptic placentae. Figure 3.4 shows the dose response and Figure 3.5 the time dependent hydrolysis of mfVSG. No significant differences were observed between specific activity values obtained from normal and pre-eclamptic preparations (13.1u ± 5.3sd, n=5 and 19.6u ± 7.1sd, n=3 respectively) (1unit = % hydrolysis/total cpm x 100 / mg prot / incubation time).

Two types of hydrolysies could have occurred: C or D type (illustration in Figure 3.6). Phospholipase C hydrolyses the phosphodiester linkage between the phosphate
and the lipid to produce a 1,2-diacylglycerol and a phosphorylated polar head group. Phospholipase D hydrolyses the phosphodiester linkage between the phosphate and the polar head group to produce phosphatidic acid (a phosphorylated DAG) and the polar head group with an exposed hydroxyl group. To determine the nature of the hydrolysis induced by microvilli preparations, the radiolabeled products were identified by TLC. Figure 3.7 shows TLC analysis of the lipidic products isolated after hydrolysis of the mfVSG. As shown in lanes 4 and 6, both PA and DAG resulted from the microvillous membrane hydrolysis of mfVSG. However, when phosphatase inhibitors were added to the reaction (lanes 5 and 7) only the PA product was detected. This suggests that the DAG has not been generated by a PLC activity but by the combined action of GPI-PLD and phosphatase activities. The phosphatase removed the phosphate of PA to form DAG as illustrated in Figure 3.6. These results showed the presence of a GPI-PLD activity in the microvilli preparation, and absence of a GPI-PLC activity.

The expression of GPI-PLD mRNA was investigated by RT-PCR using cDNA made from total RNA extracted from normal and pre-eclamptic placentae, two commercially available placental cDNA libraries, and cDNA made from total RNA extracted from the BeWo placental cell line. No expression of GPI-PLD mRNA was seen in any of the normal and pre-eclamptic placental cDNA samples (n = 4) that had previously been shown to contain β-actin mRNA. Figure 3.8.B shows the absence of GPI-PLD PCR products after RT-PCR of one normal and one pre-eclamptic sample (lanes 1 and 3 respectively). Figure 3.8.A shows that the same samples did contain β-actin. The total RNA samples were not contaminated with DNA since negative controls of the cDNA synthesis did not result in products in either PCR (lanes 2 and 4). Since a product was obtained in the GPI-PLD PCR positive control (B/lane 5), the PCR worked. Therefore, it can be concluded that GPI-PLD mRNA is not expressed in our placental samples. Similarly, no expression of GPI-PLD was detected in the placental cell line (private communication from William Hicks, University College London) and placenta cDNA libraries.
Figure 3.2: Immunohistochemical staining of GPI-PLD in sections of villi from a normal human term placenta. Frozen villi sections of a normal placenta were incubated with an anti-GPI-PLD polyclonal antibody (A) or a non immune antibody (B). Bars represent 30 µm.
Figure 3.3: Western-blotting of GPI-PLD in homogenates of placenta (lane 1) and microvilli of normal (lanes 2, 3, 4 and 6) and pre-eclamptic (lane 5) placentae. Intact placenta and both microvilli of normal and pre-eclamptic placentae show a 50 kDa band (lane 1 to 6). In addition, both microvilli of normal and pre-eclamptic placentae also show a fainter 100 kDa band (lanes 6 and 5 respectively) consistent with classical GPI-PLD expression. Homogenate of placenta and microvilli were subjected to SDS-PAGE. Following transfer to nitrocellulose, the membrane was blotted using an anti GPI-PLD antibody.
Figure 3.4: Hydrolysis of mfVSG induced by serial dilution of a placental microvilli preparation. 6 μg of [3H]mfVSG (18 000 cpm) was incubated at 37°C for 30 min in the presence of an increasing amount of membrane. Hydrolysis was measured by determining the amount of product which fractionated into the organic phase. Each point represents the mean ± SD of three different samples.
Figure 3.5: Time-dependent hydrolysis of mfVSG by placental microvilli. 6 μg of $[^3]H$mfVSG was incubated at 37°C in the presence of microvillous membrane (containing 0.25 mg) and the reaction was stopped after 15, 30 and 60 min of incubation. Hydrolysis was measured by determining the amount of product which fractionated into the organic phase.
Figure 3.6: Scheme indicating the lipidic products obtained after hydrolysis by different phospholipases. GPI-PLD hydrolysis results in phosphatidic acid (PA), PI-PLC and GPI-PLC hydrolysis results in diacylglycerol (DAG). The scheme also indicates that PA can be dephosphorylated into DAG by phosphatase.
Figure 3.7: Autoradiograph of a TLC plate demonstrating the nature of the mfVSG hydrolysis induced by placental microvillous membranes. Hydrolysis of mfVSG by GPI-PLD and PI-PLC produced PA and DAG respectively (lanes 2 and 3 respectively). Hydrolysis of mfVSG induced by microvillous membrane from normal and pre-eclamptic placentae produced both DAG and PA (lanes 4 and 6 respectively). In the presence of phosphatase inhibitors, the hydrolysis of mfVSG induced by microvillous membrane from both normal and pre-eclamptic placentae produced only PA (lanes 5 and 7 respectively). This showed that the production of DAG was not due to a phospholipase C activity but the combination of a phospholipase D and phosphatase activities. The negative control is incubation of mfVSG in reaction buffer in the absence of enzyme or microvillous membrane (lane 1).
Figure 3.8: RT-PCR for (A) β-actin and (B) GPI-PLD in normal (lane 1) and pre-eclamptic placentae (lane 3). Lanes 2 and 4 are controls for DNA contamination (no reverse transcriptase was added in the cDNA synthesis reactions) for normal and pre-eclamptic samples respectively. Lane 5 is the RT-PCR positive control (for GPI-PLD, the full-length GPI-PLD cDNA clone isolated from a human liver cDNA library; for β-actin, an aliquot of a skeletal muscle cDNA library. Lane 6 is a negative control for the PCR (no DNA was added).
DISCUSSION

In order to understand the reason(s) for the high level of IPGs and undetectable amount of GPI in pre-eclamptic placenta, and to clarify the mechanism of release of IPGs in the placenta, our study led to the investigation of GPI-PLD in this organ. The presence of GPI-PLD activity in the human placenta had already been proposed (Malik and Low 1986), the findings of this chapter gave additional information on the expression of the enzyme.

GPI-PLD, a 50 kDa protein present in the human placenta

In this study, the presence of GPI-PLD has been detected in the placenta and its size determined. This was done by immunostaining and by Western-blotting using the same polyclonal antibody.

The immunostaining indicates the presence of GPI-PLD in both the internal stroma and trophoblast. The method was not precise enough to define which types of cells of the stroma were positive and which were not. Hofbauer cells are macrophages present in the internal stroma, they may contain GPI-PLD as GPI-PLD was found in human macrophages (O'Brien et al 1999).

In addition, the size of the recognised protein has been determined by Western-blotting. The polyclonal anti-GPI-PLD antibody detected in every sample tested (homogenate of placenta, microvilli preparations of both normal and pre-eclamptic placenta) a 50 kDa protein and in some of them a lower amount of a 100 kDa protein. The intact native form of GPI-PLD is 100-120 kDa and is found in high amounts in bovine and human serum (Davitz et al 1989, Huang et al 1990). However even if the main form of GPI-PLD in the serum has a 100-120 kDa molecular mass, GPI-PLD has also been detected as a 47 kDa active protein in small amounts in mammalian serum (Hoener et al 1994). Our results suggest that the 50 kDa GPI-PLD is the main form in the placenta. This smaller protein could be the result of partial proteolytic degradation. It has been reported that trypsin treatment of bovine serum GPI-PLD generated, in vitro, three fragments of 33, 39 and 47 kDa of GPI-PLD, with the activity associated with the 39 kDa fragment (Heller et al 1994).
Presence of GPI-PLD in the syncytial microvillous membrane

This work shows that GPI-PLD is present in microvilli preparations. Plasma membrane location is consistent with the GPI-PLD immunostaining observed around the surface of meloid cells (Xie and Low 1994). The presence of GPI-PLD in microvilli does not exclude its presence in intracellular compartments and more particularly in lysosomes as proposed in the literature (Hari et al 1996).

The brush border membrane is rich in GPI-anchored proteins. Since GPI-anchored proteins are GPI-PLD substrates, the presence of GPI-PLD in microvilli suggests a possible regulation of the release of soluble proteins into the maternal circulation. Alkaline phosphatase is present in the syncytial brush border membrane. GPI-PLD has been proposed to cleave the high molecular weight, hydrophobic form (GPI-anchored form) of alkaline phosphatase into a soluble form. GPI-PLD may thus regulate the release of this protein into the circulation.

In addition to IPGs and GPI, GPI-PLD, another component of the IPG/GPI signalling machinery has therefore been detected in microvillous membrane preparations. This strongly suggests the existence of this signalling pathway in this part of the placenta with GPI-PLD as the phospholipase responsible for IPG release. In the following chapter, the proposed hydrolysis of placental GPI by GPI-PLD is examined.

Demonstration of GPI-PLD activity and not GPI-PLC activity

In this work, GPI-PLD activity was found in the membrane preparation where GPI-PLD had been detected by the anti-GPI-PLD antibody (Western-blotting experiments). However, it has not been proven that the protein recognised by the antibody was responsible for the GPI-PLD hydrolytic activity. The shortage of antibody prevented me from doing an immunoprecipitation which might have been conclusive. -Note: A monoclonal commercial anti GPI-PLD was available, but it did not recognise anything in immunostaining and in Western-blotting experiments. This can be explained by the fact that it has been raised against a denatured protein or C-terminal peptide fragment. Monoclonals are also less efficient in immunoprecipitations.

The radiolabelled GPI-anchored protein mfVSG was a good tool not only to detect phospholipase activity in the placental membrane preparations, but also to demonstrate that it was only a phospholipase D type activity by identifying the
generated radiolabelled lipidic product of the reaction. Phosphatidic acid (PA) is the lipidic product released by a phospholipase D activity, and diacylglycerol (DAG) is the lipidic product released by a phospholipase C activity. Both PA and DAG were detected after hydrolysis of mfVSG by the microvillous membrane preparation. The addition of phosphatase inhibitors to the reaction mixtures resulted in the complete disappearance of DAG. This demonstrated that the formation of DAG was due to a combination of phospholipase D and phosphatase activities instead of a phospholipase C activity. The combined action of GPI-PLD and phosphatase was also demonstrated in an extract of bovine liver using the same phosphatase inhibitors (Heller et al 1992).

**GPI-PLD activity in normal and pre-eclamptic placenta**

Immunohistochemical experiments did not show higher GPI-PLD staining in sections of pre-eclamptic placentae than in those of normal placentae. This suggests that the amount of immunoreactive placental GPI-PLD is not altered in pre-eclampsia.

A high GPI-PLD activity was found in microvilli preparations, but this may not reflect the activity under physiological conditions. In the plasma where GPI-PLD is abundant, (7μg/ml), if the enzyme were as active as measured in vitro, the amount would be sufficient to cleave most of GPI-anchor proteins within one minute (estimated by Low (Low 1990)). It is therefore obvious that the best conditions are provided for the in vitro measure of GPI-PLD activity. These optimum conditions include the presence of the detergent Nonidet P-40, which is thought to provide a better environment for the enzyme. Consequently, the absence of a difference in GPI-PLD activity, observed between normal and pre-eclamptic samples, does not mean that the GPI-PLD activity in pre-eclamptic and normal placenta is at the same level. In addition, GPI-PLD activity is modulated by environmental agents like lipids (Low and Huang 1993, Villar et al 1998). It is relevant to note that microvilli from pre-eclamptic and normal placenta were observed to be different in shape (Jones and Fox 1980) and that the shape of caveolae was reported to be related to their lipid composition (Anderson 1998). Since microvilli have been defined as tubular caveolae the abnormal club-like shape of microvilli may be due to an abnormal
lipidic composition and this could contribute to an abnormal regulation of GPI-PLD present in these structures. GPI-PLD activity has also been recently reported to be inhibited by protein kinase A induced phosphorylation and stimulated by phosphatase (Civelli et al 1999). Interestingly, a difference in phosphatase activities between normal and pre-eclamptic placentae has been observed (Jones and Fox 1980). In normal placenta, alkaline phosphatase was distributed predominantly on the microvillous plasma membrane, whereas in pre-eclamptic placenta the enzyme was spread throughout the cytoplasm. The activity of acid phosphatase was also increased in pre-eclampsia mainly in the lysosomes (Jones and Fox 1980), which are intracellular compartments where GPI-PLD has been located (Hari et al 1996). The modulation of GPI-PLD activity by several factors such as kinases, phosphatases or the lipidic environment, not taken into consideration in our in vitro assay, may therefore result in a difference in normal and pre-eclamptic placentae in vivo.

Thus, the question of the involvement of GPI-PLD in pre-eclampsia remains to be answered. A higher activity of GPI-PLD would explain higher levels of IPGs as well as a higher level of alkaline phosphatase in the circulation and lower level in the placenta and more specifically in the microvillous membrane.

The placenta does not synthesise GPI-PLD
Although the protein was detected and found to be active, no evidence of mRNA for GPI-PLD was found. The investigation was performed on different placental samples, on a trophoblast placental cell line, and also on two commercial cDNA libraries. The PCR carried out under the same conditions was positive using libraries of cDNA from liver in which GPI-PLD mRNA is expressed (Scallon et al 1991). Until now, GPI-PLD mRNA has only been found in liver (Scallon et al 1991), islets of pancreas (Metz et al 1991, Tsang et al 1992), and macrophages (O'Brien et al 1999). Our results suggest that the placenta does not produce GPI-PLD. The main tissue of the placenta is the trophoblast, but our placental cDNA samples and cDNA libraries contain not only cDNA from the trophoblast but also cDNA from all cellular types of the placental villi including mesenchyme cells, macrophages, endothelial cells and muscular cells. However, trophoblast is the main tissue and its cDNA should represent the main part of the placental cDNA. We therefore think that GPI-
PLD is not synthesised in the trophoblast of the placenta but we cannot excluded the possibility that some cells, which are in a minority in the placenta, express GPI-PLD (undetectable by PCR).

**Model of uptake of GPI-PLD**

We propose an uptake of GPI-PLD into the trophoblast from the maternal blood as a plausible explanation for the presence of GPI-PLD protein and absence of its mRNA in the placenta. The uptake of GPI-PLD has previously been observed *in vitro* into neuroblastoma cells (Hari *et al* 1997). The intact 115 kDa form and tryptic proteolytic active fragments of GPI-PLD were incorporated into the cells where they were partially degraded in lysosomes. The uptake seems plausible in the placenta because of the absence of the GPI-PLD transcript, the abundance of the protein in the maternal blood where placental villi are bathed, and because GPI-PLD is present in microvilli localised at a frontier position between the placenta and the maternal blood. The proposed GPI-PLD uptake is illustrated in Figure 3.9. On one hand, the uptake may preferentially select the 50 kDa protein, which may be present as a minority species in the human blood since a 47 kDa GPI-PLD form has been detected in minority in the bovine serum. Whereas on the other hand the major form of GPI-PLD, the 100-120 kDa form, may be uptaken and transferred into lysosomes where it is cleaved by trypsin or other proteases before delivery to the plasma membrane, as has been proposed in neuroblastoma cells. Following this hypothesis GPI-PLD present in the placenta would have a maternal origin.

This completes our investigation into the elements of the IPG/GPI signalling system showing that the GPI-PLD enzyme is present in both normal and pre-eclamptic placenta (Figure 3.10). In the next chapter I investigate whether or not GPI-PLD cleaves IPG precursor GPI to release biologically active IPGs.
Figure 3.9: Model for GPI-PLD uptake explaining the presence of a 50 kDa GPI-PLD form in the syncytiotrophoblast microvillous membrane. Abbreviations: GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; IPG, inositolphosphoglycan; STB, syncytiotrophoblast.
Figure 3.10: Hypothetical role of GPI-PLD in the model of IPG release in the microvillous membrane of normal and pre-eclamptic placenta. GPI-PLD has been detected in microvillous membrane of both normal and pre-eclamptic placentae. GPI has been detected in microvillous membrane of normal placentae but not in microvillous membrane of pre-eclamptic placentae. IPG maybe released from microvillous membrane after stimulation of GPI-PLD, which would hydrolyse GPI to generate IPGs. A complete degradation of the pool of GPI by an abnormal stimulation of GPI-PLD may explain the higher level of released IPG. Abbreviations: GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; IPG, inositolphosphoglycan; STB, syncytiotrophoblast.
Chapter 4

Hydrolysis of GPI

by GPI-PLD
**INTRODUCTION**

In addition to the well-established transmembrane signalling events including cyclic nucleotide generation, polyphosphatidylinositol hydrolysis and calcium ion fluctuation, an alternative pathway for receptor-mediated signal transduction has been proposed throughout this thesis. It is the IPG/GPI signalling system which involves the hydrolysis of a GPI by a phospholipase, thus generating biologically active IPGs.

Various compounds, thought to be the above-stated elements of this signal transduction pathway, have been detected in the human placenta and more specifically in the syncytial brush border membrane, the membrane facing the maternal blood. These are the findings of the previous chapters of this thesis and they support the existence of such a signalling system in this part of the placenta.

*IPGs in the Placenta*

The presence of IPG activity had been reported in the human placenta (Suzuki *et al* 1984, Kunjara *et al* 2000a). In Chapter 1 of this thesis, IPGs have been located in both the cytoplasm and microvillous membrane. The presence in microvilli preparations has been detected after direct extraction and measurement of activity of the extracted products in *in vitro* assays including cell proliferation of EGR T17 cells, inhibition of cAMP dependent protein kinase A and stimulation of PDH phosphatase. IPGs can also be obtained by indirect methods such as stimulating cells or membrane preparations with insulin (Saltiel and Cuatrecasas 1986, Macaulay and Larkins 1988), or hydrolysing GPI with PI-PLC (Saltiel and Cuatrecasas 1986, Mato *et al* 1987a, Merida *et al* 1988, Macaulay and Larkins 1990).

*GPI in the Placenta*

In Chapter 2, GPI, a glycolipid which has been isolated following a method used to obtain IPG precursor molecule, has also been extracted from fractions of total plasma membrane as well as micovilli preparations of normal placentae.
GPI-PLD in the Placenta

The enzyme GPI-PLD, a phospholipase specific to GPI has also been detected in microvilli preparations. The enzyme was active on mfVSG, a GPI-anchored protein of the protozoan parasite Trypanosoma brucei. The molecular weight of the identified GPI-PLD has been determined: two proteins were immunoreactive with an anti-GPI-PLD antibody, one of 50 kDa and the other of 100 kDa that seemed to be present in smaller quantities than the 50 KDa form. GPI-PLD is not synthesised by the placenta but seems to have a maternal origin. It was also demonstrated in the same chapter that there is no GPI-PLC activity in the microvilli. Thus, if the IPG/GPI signalling occurs in the placenta, GPI-PLD is the only candidate for the enzyme responsible for the hydrolysis of GPI.

GPI-PLD in the IPG/GPI Cellular Signalling System

GPI-PLD has been proposed to be the mammalian enzyme responsible for the hydrolysis of GPI and therefore, the enzyme responsible for IPG release in mammals (Rademacher et al 1994, Varela-Nieto et al 1996, Jones et al 1997, Jones and Varela-Nieto 1998).

Evidence for the involvement of GPI-PLD in IPG release includes the fact that anti-GPI-PLD antibodies block IgE-stimulated histamine release in mast cells, which is an IPG dependent event (Rademacher et al 1994). In addition Villar et al (Villar et al 1998) have shown that bovine serum GPI-PLD cleaves free GPI extracted from rat liver in artificial vesicles, the hydrolysis being dependent on the lipid environment. The fact that free GPI is a substrate for GPI-PLD has also been shown by the same group by visualising the disappearance of radiolabelled GPI after GPI-PLD treatment (Jones et al 1997). In addition, IPG-like activity was obtained after incubation of rat liver GPI with bovine serum GPI-PLD. The IPG like activities were measured by inhibition of cAMP-dependent PKA and stimulation of cell proliferation in the cochleovestibular ganglion. These IPGs were also recognised by an anti-IPG antibody (Jones et al 1997).

It is noteworthy that the latter report is the only one to show that IPG-like activity may be generated by GPI-PLD hydrolysis. In the other reports, which show generation of IPGs through phospholipase activation, the enzyme used was PI-PLC.
Furthermore, as mentioned in Chapter 2, several studies report the extraction of GPI molecules. Some of them were shown to be GPI-PLD substrates (van't Hof et al 1995, Baumann et al 2000), or PI-PLC substrates (Ralton and McConville 1998, Zawadzki et al 1998), but whether or not the phospholipase reaction on these GPIs generates IPGs remains to be demonstrated.

Hypotheses
The findings of the previous chapters supported the existence of the IPG/GPI signalling system in the placenta and more specifically in the microvillous membrane since each element has been detected. In this Chapter, the relationship of the three elements IPGs, GPI and GPI-PLD is further investigated. Thus, the two resulting hypotheses of this Chapter are firstly that placental GPI is a substrate for GPI-PLD and secondly that the GPI-PLD catalysed hydrolysis of GPI results in the production of active IPGs.

Aim of the Chapter
Extracted placental GPI were submitted to hydrolysis by GPI-PLD. The hydrolysis is checked by visualisation of GPI disappearance and detection of the generated lipid product. The detection of IPGs after GPI hydrolysis by GPI-PLD is also investigated.
MATERIALS & METHODS

The susceptibility of GPI to hydrolysis by GPI-PLD was investigated by two methods. The first was by detecting the disappearance of the substrate GPI after incubation with the enzyme GPI-PLD. The second was by detecting the lipidic product of the reaction. The final challenge was to detect IPG-like activity after the treatment of placental GPI with recombinant GPI-PLD (rGPI-PLD).

1-Detection of GPI after GPI-PLD treatment

Materials

Placental GPI was extracted as described in Chapter 2. Recombinant GPI-PLD (in 20 mM sodium phosphate, 500 mM sodium chloride) was made in the laboratory by Dr JN Schofield, University College London. Nonidet P-40 (NP-40) was purchased from ICN Biomedicals Inc (Aurora, Ohio, USA). Anisaldehyde was obtained from Sigma Aldrich Ltd (Poole, England). Iodine and HPTLC plates were obtained from BDH Ltd (Poole, England).

Method

Placental GPI (about 0.15 mg of extracted material, see Chapter 2) was incubated overnight at 37 °C with various amounts of GPI-PLD (0, 0.05, 0.1 and 0.2 units), in a final volume of 50 μl of 0.1% NP-40, 20mM Tris Maleate, 45 mM NaCl.

After incubation, 300 μl of chloroform and 300 μl of 0.1M KCl were added, and the reaction vortexed and centrifuged in order to separate the aqueous and organic products. The chloroform phase was dried, re-suspended in 10 μl of chloroform/methanol (1:1) and spotted onto a HPTLC plate for chromatography in chloroform/methanol/0.25% KCl (5/4/1 by volume). GPI was then detected by iodine vapour and anisaldehyde staining. A scanner was not available in the laboratory at the time of this experiment. Thus, in order to retain physical copy of these data, the plate was photocopied. The photocopy was then scanned for presentation in this present chapter.
2-Detection of lipidic products of the reaction

The susceptibility of GPI to hydrolysis by GPI-PLD was also determined by detecting the presence of the generated lipidic product.

Materials

Placental GPI was extracted as described in Chapter 2. rGPI-PLD was as before from Dr JN Schofield, University College London. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), dithiothreitol and alkaline phosphatase were purchased from Sigma Aldrich Ltd (Poole, England). $[^32P]$-gamma-ATP was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Thin layer chromatography (TLC) plates were Silica gel 60 obtained from BDH Ltd (Poole, UK).

$sn-1,2$-diacylglycerol assay kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The kit contained 50 nmol $sn-1,2$-diacylglycerol, a preparation of E. Coli diacylglycerol kinase in 10mM potassium phosphate buffer pH7.0 containing 20% (v/v) glycerol and 2 mM mercaptoethanol, an enzyme diluent solution containing 0.01M imidazole/HCl and 1mM diethylenetriaminepentaacetic acid buffer pH6.6, 0.1M imidazole/HCl buffer pH6.6 containing 0.1M NaCl, 25 mM MgCl$_2$ and 2 mM ethylene glycol-bis (beta-aminoethyl ether) N,N-tetraacetic acid (EGTA), and a detergent solution containing 7.5% (w/v) n-octyl-Beta-glucopyranoside and 5 mM cardiolipin in 1 ml diethylenetriaminepentaacetic acid.

Method

The hydrolysis of placental GPI was carried out as described before except that buffer did not contain NP-40 but PE (about 0.01 mg) and PC (about 0.02 mg) (These quantities were arbitrarily chosen). Alkaline phosphatase (5 μl of a solution containing 0.4 units in 10 mM Tris-HCl pH 7.0 (one unit hydrolyses 1.0 μmole of p-nitrophenylphosphate per min at pH 10.4 at 37°C)) was also added to the reaction mixture in order to dephosphorylate the product of the reaction PA into DAG, which can be detected using the commercial kit.

The basis of the DAG assay procedure is a radioenzymatic assay employing the enzyme diacylglycerol kinase, which converts DAG to $[^32P]$phosphatidic acid in the presence of $[^32P]$ATP. The detection of $[^32P]$ is achieved by TLC.
After hydrolysis of GPI, organic and aqueous products were separated; chloroform fractions were tested for presence of DAG. A volume of 200 μl of the chloroform phases and standards containing 0 or 500 pmol of DAG were dried using a SpeedVac concentrator (Stratech Scientific, Luton, UK). To the dried standard/samples, 20 μl of detergent was added. After mixing by vortexing and sonicating for 3 min in water bath, 70 μl of a ‘reagent mix’ solution containing DAG kinase, DTT was added and the enzyme reaction was initiated by adding 10 μl of [³²P]-ATP (0.1 μCi). After 30 min incubation at room temperature, the reaction was stopped by adding 20 μl of 1 % (v/v) perchloric acid and 450 μl of chloroform/methanol (1:2). Tubes were mixed, left for 10 min and centrifuged at 2000 g for 1 min. 150 μl chloroform and 150 μl 1 % perchloric acid were added, and centrifuged after thorough mixing. After centrifugation, the upper phase was discarded and the lower chloroform phase was washed twice with 2 × 1 ml of 1 % perchloric acid.

The presence of phosphatidic acid was detected by TLC. The lower phase was transferred into new tubes, dried by with the SpeedVac concentrator and loaded onto TLC plates and running in solvent containing chloroform/methanol/water/ammonia (9/6/0.5/0.35 by volume). The plates was dried for a few minutes and placed in cassettes overnight at −80°C with autoradiographic film.

3- Measure of IPG-like activity

Materials
Placental GPI was extracted as explained in Chapter 2. rGPI-PLD was made in the laboratory by Dr JN Schofield, University College London. Nonidet P-40 (NP-40) was obtained from ICN Biomedicals Inc (Aurora, Ohio, USA).

Method
The hydrolysis of placental GPI was carried out as described before with some modifications. GPI-PLD was previously transferred from phosphate buffer to hydrolysis reaction buffer (20 mM Tris Maleate, 0.1% NP-40, 45 mM NaCl pH 7.0) by dialysis since the phosphate buffer may interfere in assays measuring IPG-like activities. The amount of rGPI-PLD was about 0.4 units. Equivalent of 0.25 and 0.125 mg of placental GPI were used. (An aliquot of 1mg of GPI was suspended in
methanol and the volume required was dried in the reaction tubes). The total volume of the reaction was 25 μl. Controls with buffer only, with GPI and buffer, and with GPI-PLD and buffer were performed. After the overnight incubation at 37°C, IPG-like activity (stimulation of PDH phosphatase and inhibition of cAMP-dependent PKA) was directly tested on 5 μl of this reaction mix and on water and organic phases (inhibition of cAMP-dependent PKA only) after extraction of the remaining 15 μl reaction mixture with 300 μl of Chloroform and 300 μl of 0.1M KCl. The water phase was subjected to a second extraction with water-saturated butanol and 0.1M KCl as before and both aqueous and organic fractions were tested for the IPG-like activity (both stimulation of PDH phosphatase and inhibition of cAMP-dependent PKA). The stimulation of PDH phosphatase and inhibition of cAMP dependent protein kinase A were measured as described in Chapter 1. When the assay was performed on the reaction buffer, an aliquot of 5μl was tested. When the assay was performed on the water and organic phases, samples were dried and reconstituted in a volume such that the 5 μl tested was comparable to the 5 μl of the reaction mix.

A sample of 0.05 mg GPI freshly suspended in 5 μl reaction buffer was also tested on inhibition of cAMP dependent protein kinase A. A control consisting of reaction buffer only was tested and found to be negative.
RESULTS

Disappearance of GPI after GPI-PLD treatment

The disappearance of GPI after GPI-PLD treatment has been visualised on HPTLC plates and is represented in Figure 4.1. As seen, GPI is still detectable when incubated with 0.05 and 0.1 unit of GPI-PLD (lanes 4 and 5 respectively), although the amount present is reduced. However when incubated in the presence of 0.2 units GPI-PLD, all detectable GPI has disappeared (lane 6). In lane 1, GPI-PLD and GPI were not added in the reaction mixture. In lane 2, GPI-PLD was added but GPI was not added in the reaction mixture. And, in lane 3, GPI was added but GPI-PLD was not added in the reaction mixture. The experiment was performed twice and was found to be reproducible. This result shows that GPI has been hydrolysed by GPI-PLD.

Detection of DAG after placental GPI treatment with GPI-PLD

The cleavage of GPI by GPI-PLD results in the production of PA, which is expected to be converted into DAG when alkaline phosphatase is added to the reaction mixture. We examined whether rGPI-PLD can cleave extracted placental GPI by detecting the lipidic product. DAG is detected using a method which converts DAG into $^{32}\text{PA}$ in the presence of DAG kinase and $[^{32}\text{P}]\text{ATP}$. The radiolabelled product, $^{32}\text{PA}$, is then detected by TLC. Figure 4.2 indicates that DAG has been generated when GPI has been incubated with recombinant GPI-PLD in the presence of alkaline phosphatase. $^{32}\text{PA}$ has been detected with a Rf = 0.3 in samples where GPI and GPI-PLD were incubated in the presence of alkaline phosphatase (lane 8 and 9). $^{32}\text{PA}$ has not been detected in samples without GPI or GPI-PLD (lane 6 and 7 respectively) or when the reaction was carried out in the absence of alkaline phosphatase (lanes 4 and 5). Lane 1 is the negative control of the DAG phosphorylation (no DAG) reaction and lane 2 the positive control (500pmol of DAG).

Generation and assessment of an IPG-like activity from extracted placental GPI treated with GPI-PLD

To detect IPG like activity, the inhibition of cAMP-dependent-PKA and the stimulation of PDH phosphatase were assayed after incubation of GPI with GPI-
PLD. Figure 4.3 shows PKA and PDH phosphatase activity of 5 μl of the reaction mixture. As seen, samples in which GPI-PLD was added to GPI (columns 4 and 5) were active. However, it can not be concluded that GPI has been hydrolysed by GPI-PLD to generate IPGs since the control consisting of GPI only was also positive in both inhibition of PKA and stimulation of PDH phosphatase (columns 2). The activity found in sample 2 could have been due to GPI itself or to the degradation of GPI during the overnight incubation. The following step was therefore to measure the activity of GPI before incubation. About 0.05 mg of GPI was then found to induce 81 % inhibition of cAMP-dependent PKA, which is about the same activity found after incubation. This result suggests that GPI contained the IPG-like activity.

Although GPI was found to be active, IPGs may have been released. To separate IPGs from GPI, the 20 μl of remaining reaction mix was subjected to an extraction procedure. Both organic and water fractions were tested for IPG-like activities. The chloroform fractions of all samples were negative (data not shown) whereas, as seen in Figure 4.4, PKA inhibition activity was found to fractionate in the water fractions of samples in which activity was found before the extraction, including sample 2, the GPI control (PDH activity was not determined on these samples). To ensure that the extraction was efficient, a second extraction was carried out on the remaining water fraction using water-saturated butanol as organic solvent. The activity on both inhibition of PKA and stimulation of PDH phosphatase of both water and butanol fractions is shown in Figure 4.5. As before, activity was found in the water fraction in all samples where GPI had been added (columns 2, 4 and 5). This suggested that either GPI fractionates in the water fraction, or that the GPI extract contains water-soluble compounds responsible for IPG activity.

Although low, IPG activity was found in butanol fractions in samples where GPI was added. These IPG-like activities seemed to be related to the amount of GPI present in the samples. As seen on the figure, this observation is especially pronounced when determining the activation of PDH phosphatase. In samples where 0.25 mg of GPI was added, the percentage of activation of PDH phosphatase induced was 117 and 155 for samples 2 and 4 respectively whereas when 0.12 mg of GPI was added, the percentage of activation of PDH phosphatase was 42.
Figure 4.1: Representation of HPTLC of GPI after GPI-PLD treatment. GPI present in the absence of GPI-PLD (lane 3) is readily detectable. Incubation with GPI-PLD reduces gradually the amount of GPI seen (lanes 4 to 6). Incubation with 0.2 units of GPI-PLD results in complete disappearance (lane 6). Extracted placental GPI was incubated overnight at 37°C in the absence (lane 3) or presence of 0.05, 0.1 and 0.2 units of rGPI-PLD (lane 4, 5 and 6 respectively). Negative controls were: reaction buffer without GPI and GPI-PLD (lane 1), and reaction buffer with GPI-PLD but without GPI (lane 2). Organic products were fractionated by chloroform extraction, loaded onto HPTLC plates and developed in chloroform/methanol/0.25%KCl (5/4/1 by volume). GPI was revealed by iodine and anisaldehyde staining.
Figure 4.2: TLC of $[^{32}\text{P}]$PA for detection of DAG after placental GPI incubation with GPI-PLD in the presence of alkaline phosphatase. DAG was detected when GPI was incubated with GPI-PLD in the presence of alkaline phosphatase (lanes 6 and 7). About 0.15 mg of GPI extract were incubated with rGPI-PLD. Controls were reaction buffer only (lanes 3 and 6), reaction buffer with GPI but without GPI-PLD (lanes 4 and 7). The incubation was done in the absence (lanes 3, 4 and 5) and presence of alkaline phosphatase (lanes 6 to 9). After chloroform extraction, the organic phase was tested for the presence of DAG by REA. Lane 1 is the negative control for REA in which DAG was not added in the reaction buffer. Lane 2 is the positive control for REA in which 500 pmole of standard is present in the reaction buffer.
Figure 4.3: IPG-like activity in the reaction mixture after GPI hydrolysis by GPI-PLD. Both inhibition of cAMP-dependent PKA and stimulation of PDH phosphatase were detected in samples containing GPI and GPI-PLD (columns 4 and 5) and in a sample containing GPI only (column 2). The inhibition of cAMP-dependent PKA and the stimulation of PDH phosphatase were measured in the reaction mixture after incubation of placental GPI with GPI-PLD. rGPI-PLD was incubated overnight at 37°C in the absence (columns 3) and presence of 0.25 and 0.12 mg of extracted placental GPI (columns 4 and 5 respectively). Controls consisted of reaction buffer (columns 1), reaction buffer with 0.25 mg of GPI only (columns 2) and reaction buffer with GPI-PLD only (columns 3).
Figure 4.4: Measure of cAMP-dependent PKA inhibition induced by water phases obtained by fractionation of samples in which GPI has been incubated with GPI-PLD for hydrolysis. Activity was detected in samples containing GPI and GPI-PLD (columns 4 and 5) and in the sample containing GPI only (column 2). Fractionation was carried out with chloroform and 0.1M KCl once rGPI-PLD was incubated in the absence (column 3) and presence of 0.25 and 0.12 mg of extracted placental GPI (columns 4 and 5 respectively). Controls samples consisted of the water fractions of reaction buffer (column 1), reaction buffer with 0.25 mg of GPI only (column 2) and reaction buffer with GPI-PLD only (column 3).
Figure 4.5: Assay of IPG-like activities on both aqueous and organic phases after butanol fractionation of samples in which GPI has been incubated with GPI-PLD. Columns A and B represent assays on aqueous and butanol fractions respectively. Both inhibition of cAMP-dependent PKA and stimulation of PDH phosphatase were detected in the aqueous fraction of samples containing GPI and GPI-PLD (columns 4A and 5A) and in the sample containing GPI only (column 2A). Activities are lower but are also found in butanol fractions (columns 2B, 4B and 5B). Fractionation was carried out on the water fraction of a first extraction performed with chloroform on samples consisting of rGPI-PLD incubated in the absence (columns 3) and presence of 0.25 and 0.12 mg of extracted placental GPI (columns 4 and 5 respectively). Controls samples consisted of reaction buffer (columns 1), reaction buffer with 0.25 mg of GPI only (columns 2) and reaction buffer with GPI-PLD only (columns 3).
DISCUSSION

Findings
The work presented in this Chapter strongly suggests that extracted placental GPI is a substrate for rGPI-PLD. This is consistent with the proposal stated in the previous Chapters, that an IPG/GPI signalling system exists in the placenta involving a GPI-PLD enzyme.

It is noteworthy, that the fact that the extracted placental GPI has been hydrolysed by a GPI specific phospholipase, confirms the nature of the GPI molecule, since by definition GPI-PLD only hydrolysies molecules with GPI moieties, including free-GPI and GPI-anchored proteins.

Two Methods
The hydrolysis of extracted placental GPI by GPI-PLD has been demonstrated by two methods. The first is by visualisation of the disappearance of the substrate and the second is by visualisation of the appearance of the products. The first method requires high amounts of GPI and enzyme. The visualisation has been performed with approximately 0.15 mg of extracted GPI (corresponding approximately to 0.5 g of placenta) and the disappearance was observed with 0.1 and 0.2 units of GPI-PLD. As explained in the Materials and Methods Section, a scanner was not available in the laboratory at the time of this experiment. To keep these data, the plate was photocopied and the photocopy was then scanned for presentation in the present thesis. This experiment has been done twice and the result has been reproducible.

Jones et al (Jones et al 1997) also show the hydrolysis of GPI by GPI-PLD by visualising the disappearance of the substrate. The human liver GPI in their investigation was previously radiolabelled, the results were therefore visualised by autoradiography and were thus more sensitive.

The advantage of detecting radiolabelled compounds was used in Method 2 by radiolabelling DAG with DAG kinase in the presence of $[^{32}P]ATP$. The product released by GPI-PLD hydrolysis is not DAG but PA. However, treatment of PA by alkaline phosphatase converts PA into DAG. Thus, to demonstrate that GPI has been cleaved by GPI-PLD, this second method consists of detecting DAG which is generated by GPI hydrolysis by GPI-PLD in the presence of alkaline phosphatase.
Possible modulation of GPI-PLD activity by phosphatidylcholine/phosphatidyl-ethanolamine (PE/PC) and detergent

In the second method (DAG assay), the experiment was performed in the presence of phosphatidylethanolamine and phosphatidylcholine. These conditions were chosen because the hydrolysis of GPI seems to be better in the presence of phosphatidylethanolamine and phosphatidylcholine, lipids which are not substrates of GPI-PLD but which were previously shown to modify the activity of GPI-PLD in artificial vesicles (Villar et al 1998). As already reported in the Introduction of Chapter 3, in this latter report, it was shown that bovine liver GPI-PLD hydrolyses 81% of GPI in vesicles made of GPI / phosphatidylcholine / phosphatidylethanolamine with a ratio of 1:1:2, whereas in the presence of phosphatidylethanolamine and sphingomyelin (GPI / phosphatidylethanolamine / sphingomyelin with a ratio of 1:2:1), GPI-PLD activity dropped to 14% (Villar et al 1998).

In the first method (HPTLC visualisation), PE and PC were not included but detergent was added to the reaction buffer. Detergent and PE/PC may have a similar role toward the activity of GPI-PLD.

Detergent is required to assay GPI-PLD activity when the substrate is a GPI-anchored protein (Low 1990, Low and Huang 1991). Without detergent the activity of the enzyme toward these GPI-anchored proteins can not be detected. It would be interesting to see if the substitution of detergent by a mixture of PE/PC can modify GPI-PLD activity towards GPI-anchored protein hydrolysis.

The group which published the hydrolysis of GPI in artificial vesicles containing PE/PC is also the group which published the hydrolysis of radiolabelled GPI visualised by TLC referred to above. In this latter report, the hydrolysis of [$^3$H]GPI was carried out not in the presence of PE/PC, but as Scallon et al (Scallon et al 1991) did, when GPI-PLD activity was measured on [$^3$H]mVSG, ie using detergent (0.2% NP40) in the buffer. Therefore, as observed in the present work, the Spanish group obtains rat liver GPI hydrolysis by GPI-PLD either in the presence of PE/PC or detergent.

However, in the paper in which the hydrolysis of radiolabelled GPI was visualised by TLC, it has also been reported that non-radiolabelled GPI was treated by GPI-PLD
under the same conditions but in the absence of detergent and for a longer incubation
time (overnight instead of 2h30). The hydrolysis of GPI could not be demonstrated
under these conditions because GPI was not radiolabelled. However, the authors
reported the production of IPGs from this reaction. The production of IPGs will be
discussed later.

**IPG/GPI signal transduction in the placenta**

The finding that placental GPI has been cleaved by GPI-PLD, added to the results of
previous chapters, is consistent with the proposal that a cellular signalling system
involving GPI hydrolysis by a GPI-PLD enzyme exists in the microvillous
membrane of the placenta. To support the proposal, it would have been more
thorough to show the production of IPGs. The production of IPGs resulting from the
hydrolysis of GPI by GPI-PLD has not been proven in this study, but Jones and
colleagues (Jones *et al* 1997) did report such a result. The production of IPGs was
obtained after treatment of rat liver GPI with bovine serum GPI-PLD. The reaction
was carried out as described by Scallon *et al* (Scallon *et al* 1991) but without
detergent and for a longer incubation time (overnight instead of 30 min). Jones and
colleagues wrote in the Materials and Methods Section that 'IPG was prepared by
incubation of rat liver with GPI-PLD, in the absence of detergent, overnight as
previously described' and gave a reference corresponding to Scallon and colleagues’
report. However, Scallon *et al* did not measure IPG activity but studied GPI-PLD
activity on GPI-anchored proteins. Thus, Jones *et al* have not given an indication of
how IPG samples were prepared, whether or not the measure of activity was
performed directly on the reaction buffer or whether or not an extraction step
preceded the activity measurement.

In the present work, IPG activity was first measured directly in the reaction buffer.
The samples were found to stimulate PDH phosphatase and to inhibit cAMP-
dependent protein kinase A. However, a release of IPG from the hydrolysis of GPI
by GPI-PLD could not have been concluded since the negative control consisting of
GPI in the absence of GPI-PLD was also positive. This led to two hypotheses: either
GPI has been degraded during the incubation time generating IPGs (even if GPI-PLD
was not added), or GPI is active by itself. GPI was therefore tested itself and has
been shown to be active. An extraction procedure has therefore been carried out in
order to separate GPI from possible released IPGs. The IPG-like activity was found in the water fraction in the sample corresponding to the negative control (GPI without GPI-PLD). Water-soluble IPGs may be present in the GPI extract and explain the results. However since IPG-like activity has also been found in the butanol fraction, it is more probable that GPI itself is biologically active and fractionates in the water fraction. The solubility in water would be certainly due to the undefined polar head group of GPI and micelle formation of this molecule.

In addition, the fact that extracted GPI possessed IPG activity confirms that the extracted GPI is the precursor molecule of IPGs or at least a related IPG molecule.

In support of this finding, data from other researchers in our group has also shown that GPI possesses both \textit{in vivo} and \textit{in vitro} IPG-like biological activity (personal communication from K. Elased, Rademacher Group Limited, London).

In the report of Jones \textit{et al} in which IPG activity was assessed after GPI-PLD treatment of rat liver GPI, the authors did not mention that GPI could contain an IPG-like activity. They mentioned that a mock preparation of IPG, which did not contain rat liver GPI had been tested but they did not indicate if a mock preparation of IPG, which did contain rat liver GPI but not GPI-PLD, had been tested. It maybe possible that the IPG-like activity they obtained is not due to IPGs but GPI.

It should be emphasised that, according to these results, the role of GPI-PLD would be not to activate molecules but to convert molecules which already contain biological activity into soluble forms. The release of these soluble forms would of course greatly increase their potential to activate biological processes.

Similarly to what stated in the previous chapter, GPI-PLD activity measured \textit{in vitro} in these experiments may not reflect \textit{in vivo} conditions. The presence of detergent and phospholipids contributes to optimise GPI-PLD activity.

\textit{Summary}

The previous Chapters demonstrate the presence of both free GPI and GPI-PLD in the placenta and more precisely in the microvillous membrane. In this Chapter, the extracted free GPI has been shown to be a substrate for the enzyme. This finding is consistent with the existence of an IPG/GPI signalling in the microvillous membrane.
Conclusion
CONCLUSION

The work of this thesis follows on from the work of Kunjara et al (Kunjara et al 2000a) in which a relationship between IPGs and the pre-eclamptic disorder was established. In the present work the location of IPGs has been investigated in the placenta and its mechanism of release has been studied in the syncytiotrophoblast microvillous membrane in order to understand the high level of IPGs found in pre-eclampsia.

In Chapter 1 the extraction of IPGs from microvillous membrane preparations is consistent with the hypothesis that IPGs are associated with the shed fragments of the syncytiotrophoblast membrane. The shedding of the microvillous membrane is amplified in pregnancy complicated by pre-eclampsia (Knight et al 1998) and the factor(s) responsible for the endothelial dysfynction of pre-eclampsia is believed to be a component of these shed microvilli fragments (Kertesz et al 1999). A toxic effect of IPGs on endothelial cells remains to be demonstrated if we are to consider IPGs as possible factors responsible for pre-eclampsia. For this, it would be interesting to test IPGs in the model used by the group in Oxford (Kertesz et al 1999, Smarason et al 1993), in which syncytiotrophoblast membrane inhibits the proliferation of freshly isolated human umbilical vein endothelial cells. The method measures the inhibition of proliferation of these cells after incubation with the samples, by measuring the incorporation of radiolabelled thymidine.

The hypothesis, that IPGs are associated with the shed fragments, explains the higher levels of IPGs in urine, since there would be higher circulating IPG levels. It does not explain however the high levels of IPGs in the placenta as observed in immunostaining as well as by extraction of both homogenates of placenta (Kunjara et al 2000a) and microvilli (present work chapter1).

The second hypothesis proposed involves an abnormal release of IPGs from the placenta and more specifically from this microvillous membrane.

The presence of IPGs in placenta and in microvillous membrane preparations suggests the existence of the cellular signalling system which involves IPGs as second messengers in this organ and more specifically in the microvillous membrane. The presence of insulin receptors in this membrane and the fact that IPGs
were found to mimick insulin in human placental steroidogenesis, supports the existence of such a signalling system. It is noteworthy that IPGs have been proposed to be not only messengers of insulin but also of other hormones and growth factors such as prolactin, IGF-I and hCG, which all have important functional roles in the placenta (Spellacy et al 1967, Simpson and MacDonald 1981, Siler-Khodr et al 1991, Gluckman and Harding 1997, Muyan and Boime 1997, Bauer et al 1998, Petraglia et al 1998).

A defect in the mechanism of IPG release has been suspected in pre-eclampsia. This has led to the investigation of elements of the signalling system involving GPI hydrolysis/IPG release in the placenta and more specifically in microvilli.

The well-established mechanism of IPG release (Rademacher et al 1994, Varela-Nieto et al 1996, Jones and Varela-Nieto 1998) consists of the ligation of a hormone to its membrane-bound receptor inducing a downstream activation, by an unknown mechanism, of a phospholipase which hydrolyses a glycolipid GPI to produce soluble IPGs. Thus, the next steps of this thesis were to investigate GPI and GPI-phospholipase in the placenta and more specifically in the microvillous membrane.

In Chapter 2, a glycolipid has been extracted from human term placenta following a method which extracts GPI, the IPG-precursor molecule from liver. It has been extracted from the plasma membrane of total placenta as well as microvillous membrane preparations. This result supports the existence of the IPG/GPI cellular signalling system in the placenta and more specifically in microvilli. Interestingly, this glycolipid was not detected in pre-eclamptic samples. This finding supports the hypothesis that a defect in the signalling system exists in pre-eclampsia.

The undetectable amount of GPI may be due to a defect of its biosynthetic pathway or an abnormal catabolism of the entire pool of GPI. This latter event could have been due to an abnormal stimulation of the phospholipase responsible for the hydrolysis of the glycolipid. This hypothesis would also explain the higher level of IPGs seen in pre-eclamptic placenta. Thus, the next step was to investigate the phospholipase thought to be responsible for this hydrolysis. A GPI-specific phospholipase has been detected and it has been demonstrated that it is not a GPI-PLC but a GPI-PLD. This enzyme was present in microvilli in two forms, a 50 kDa
and a 100 kDa form. It has been proposed that the 50 kDa form is a proteolytic fragment of the 100 kDa form. Since GPI-PLD has not been found to be synthesised by the placenta, a model has been proposed in which placental GPI-PLD has a maternal origin. It has been taken up from maternal blood in which the placental villi are bathed and in which the concentration of the 100 kDa GPI-PLD is high (7μg/ml). The enzyme transits into a lysosome compartment where it is proteolytically processed into a 50 kDa form. The model was previously proposed for neuroblastoma cells (Hari et al 1997), cells which, like the placenta do not synthesise GPI-PLD. This model is also consistent with the location of GPI-PLD in myeloid cells, in which GPI-PLD is found at the cell surface as well as in post-Golgi vesicles (Xie and Low 1994).

The activity of GPI-PLD was measured in both normal and pre-eclamptic microvilli preparations using an assay which requires detergent. No significant differences were found. However, the conditions of the assay (presence of detergent) are not physiological and perhaps GPI-PLD activity is different in normal and pre-eclamptic placentae, but this difference is not detectable in vitro. To support this, it is well known that the lipidic environment affects the activity of the enzyme (Low and Huang 1993, Villar et al 1998), and it might be possible that normal and pre-eclamptic microvilli have a different lipidic composition. Phosphorylation of the enzyme also modulates its activity, and the alkaline phosphatase content of microvilli is different in normal and pre-eclamptic placentae (Jones and Fox 1980).

Thus, the presence of a GPI-specific phospholipase D capable of generating IPGs has been located in the placenta. The involvement of GPI-PLD in pre-eclampsia has not been proven in this thesis but a role of GPI-PLD in pre-eclampsia is not excluded.

Nevertheless, the presence of GPI-PLD in microvilli suggests its involvement in the signalling system involving GPI hydrolysis and IPG release. In Chapter 4, the extracted placental GPI is shown to be a substrate for rGPI-PLD and the release of IPGs is suggested by the disappearance of GPI after GPI-PLD treatment but remains to be demonstrated. To demonstrate that IPGs are released from extracted placental GPI after GPI-PLD treatment, it seems necessary to use a technique which recognises IPGs but discriminates IPGs from GPI. Thus, techniques based on detection of IPG-like activities should be used with caution since GPI also seems to
contain such activities. A possible technique may involve an antibody specific to IPGs but which does not recognise GPI. It is noteworthy that fractionation of organic and water-soluble products of a mixture of GPI and IPGs does not obviously end in a fraction of organic products containing GPI and a fraction of water soluble products containing IPGs since the precise chemical composition of IPGs and GPI is not defined. It may be possible that the glycan part of GPI is polar enough to confer water solubility of this molecule.

A recurring theme of this thesis has been the comparison of microvilli with caveolae. Microvilli were defined as tubular caveolae by Anderson (Anderson 1998). These tubular caveolae along with caveolae of other cellular types seem to be the sites for IPG/GPI signalling.

The question of the involvement of IPGs as 'cause' or 'consequence' in the pre-eclamptic disorder remains to be answered. This is also the case for numerous other compounds which are altered in pre-eclampsia and therefore the factor(s) responsible for the disorder remains to be identified. Recently, a new compound, neurokinin B, has been found to be more abundant in the circulation of pre-eclamptic women (Page et al 2000). This neurotransmitter is believed to be a good candidate for causing the main symptom of pre-eclampsia, namely hypertension. Neurokinin B acts like many hormones and growth factors by binding to membrane receptors. It is possible that the signal transduction of neurokinin B involves the hydrolysis of membrane glycolipid and release of IPGs. Even if there is no evidence to explain their role in pre-eclampsia, IPGs and neurokinin B can be very useful to enable early diagnosis of the disorder which affects 5 to 10% of all pregnant women.

Futures Directions:
Test IPG toxic effect in pre-eclampsia, using the model of the proliferation of human umbilical vein endothelial cells.
Measure GPI-PLD activity in normal and pre-eclamptic placenta in more physiological conditions (taking into consideration the real lipidic environment and effect of phosphatase and kinase on GPI-PLD).
Measure GPI-PLD in placental cells in normal and hypoxic conditions.
Confirm GPI-PLD uptake model in the placenta using placental cells and radiolabelled GPI-PLD.
Detect IPG but no GPI after GPI hydrolysis using antibody specific to one of them.
References
REFERENCES


Arkwright PD, Rademacher TW, Dwek RA, Redman CW (1993), Pre-eclampsia is associated with an increase in trophoblast glycogen content and glycogen synthase activity, similar to that found in hydatidiform moles, *J. Clin. Invest.* 91: 2744-2753


dipeptidase. Comprehensive structural studies on the porcine anchor and interspecies comparison of the glycan core structures, *J. Biol. Chem.* 270: 22946-22956


Deeg MA, Verchere CB (1997), Regulation of glycosylphosphatidylinositol-specific phospholipase D secretion from beta TC3 cells, *Endocrinology* 138: 819-826


Field MC (1997), Is there evidence for phospho-oligosaccharides as insulin mediators?, *Glycobiology* 7: 161-168


Gottschalk WK (1991), The pathway mediating insulin's effects on pyruvate dehydrogenase bypasses the insulin receptor tyrosine kinase, *J. Biol. Chem.* 266: 8814-8819


Jalink K, van Corven EJ, Moolenaar WH (1990), Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca2(+) -mobilizing stimulus for fibroblasts. Evidence for an extracellular site of action, *J. Biol. Chem.* 265: 12232-12239

Jarett L, Seals JR (1979), Pyruvate dehydrogenase activation in adipocyte mitochondria by an insulin-generated mediator from muscle, *Science* 206: 1407-1408

Jones CJ, Fox H (1976), An ultrahistochemical study of the distribution of acid and alkaline phosphatases in placentae from normal and complicated pregnancies, *J. Pathol.* 118: 143-151


Lazar DF, Knez JJ, Medof ME, Cuatrecasas P, Saltiel AR (1994), Stimulation of
glycogen synthesis by insulin in human erythroleukemia cells requires the synthesis

LeBoeuf RC, Caldwell M, Guo Y, Metz C, Davitz MA, Olson LK, Deeg MA (1998),
Mouse glycosylphosphatidylinositol-specific phospholipase D (Gpld1) characteri-
zation, Mamm. Genome 9: 710-714

Leon Y, Vazquez E, Sanz C, Vega JA, Mato JM, Giraldez F, Represa J, Varela-Nieto
I (1995), Insulin-like growth factor-I regulates cell proliferation in the developing
inner ear, activating glycosyl-phosphatidylinositol hydrolysis and Fos expression,
Endocrinology 136: 3494-3503

Li JY, Hollfelder K, Huang KS, Low MG (1994), Structural features of GPI-specific
phospholipase D revealed by proteolytic fragmentation and Ca$$^{2+}$$ binding studies, J.
Biol. Chem. 269: 28963-28971

Librach CL, Werb Z, Fitzgerald ML, Chiu K, Corwin NM, Esteves RA, Grobelny D,
Galardy R, Damsky CH, Fisher SJ (1991), 92-kD type IV collagenase mediates

stimulation of pyruvate dehydrogenase phosphatases, Arch. Biochem. Biophys. 296:
170-174

Lippincott-Schwartz J, Donaldson JG, Schweizer A, Berger EG, Hauri HP, Yuan LC,
Klausner RD (1990), Microtubule-dependent retrograde transport of proteins into the
ER in the presence of brefeldin A suggests an ER recycling pathway, Cell 60: 821-
836

Loke YW, King A (1996), Human trophoblast development, in Human implantation:

Low MG (1990), Degradation of glycosyl-phosphatidylinositol anchors by specific
phospholipases, in Molecular and cell biology of membrane proteins: glycolipid
anchors of cell surface proteins, ed. Turner AJ, p 35-63

Low MG, Huang KS (1993), Phosphatidylic acid, lysophosphatidic acid, and lipid A are inhibitors of glycosylphosphatidylinositol-specific phospholipase D. Specific inhibition of a phospholipase by product analogues?, *J. Biol. Chem.* 268: 8480-8490


Macaulay SL, Clark S, Larkins RG (1992), Correlation of insulin receptor level with both insulin action and breakdown of a potential insulin mediator precursor; studies in CHO cell-lines transfected with insulin receptor cDNA, *Biochim. Biophys. Acta* 1134: 53-60


Malik AS, Low MG (1986), Conversion of human placental alkaline phosphatase from a high Mr form to a low Mr form during butanol extraction. An investigation of
the role of endogenous phosphoinositide-specific phospholipases, *Biochem. J.* 240: 519-527


Moran P, Caras IW (1994), Requirements for glycosylphosphatidylinositol attachment are similar but not identical in mammalian cells and parasitic protozoa, *J. Cell Biol.* 125: 333-343

Muyan M, Boime I (1997), Secretion of chorionic gonadotropin from human trophoblasts, Placenta 18: 237-241


Nestler JE, Romero G, Huang LC, Zhang CG, Larner J (1991), Insulin mediators are the signal transduction system responsible for insulin's actions on human placental steroidogenesis, Endocrinology 129: 2951-2956


Pattillo RA, Gey GO, Delfs E, Mattingly RF (1968), Human hormone production in vitro, Science 159: 1467-1469


Ralton JE, McConville MJ (1998), Delineation of three pathways of glycosylphosphatidylinositol biosynthesis in Leishmania mexicana. Precursors from different pathways are assembled on distinct pools of phosphatidylinositol and undergo fatty acid remodeling, J. Biol. Chem. 273: 4245-4257


Redline RW, Patterson P (1995), Pre-eclampsia is associated with an excess of proliferative immature intermediate trophoblast, Hum. Pathol. 26: 594-600

Redman CW (1991), Current topic: pre-eclampsia and the placenta, Placenta. 12: 301-308

Redman CW (1997), Cytotrophoblasts: masters of disguise, Nat. Med. 3: 610-611


Represa J, Avila MA, Romero G, Mato JM, Giraldez F, Varela-Nieto I (1993), Brain-derived neurotrophic factor and neurotrophin-3 induce cell proliferation in the cochleovestibular ganglion through a glycosyl-phosphatidylinositol signaling system, Dev. Biol. 159: 257-265


Rothberg KG, Ying YS, Kamen BA, Anderson RG (1990), Cholesterol controls the clustering of the glycoprophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate, J. Cell Biol. 111: 2931-2938


195


Villalba M, Alvarez JF, Russell DS, Mato JM, Rosen OM (1990), Hydrolysis of glycosyl-phosphatidylinositol in response to insulin is reduced in cells bearing kinase-deficient insulin receptors, *Growth Factors* 2: 91-97


