CHARACTERISATION OF THE MOUSE GLYCOSYL PHOSPHATIDYLINOSITOL-PHOSPHOLIPASE D (GPI-PLD) GENE.

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ABSTRACT

Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) is a serum protein presumed to cleave GPI-anchored proteins on the cell surface. As the initial characterisation of GPI-PLD suggested that more than one gene coded for this enzyme, the work described in this thesis characterised the mouse GPI-PLD gene. A full-length clone isolated from a mouse liver cDNA library was sequenced. Although it showed high homology with the recently reported sequence from an islet cell-derived GPI-PLD gene, a combination of Southern blot and RNAse protection assays demonstrated that only one GPI-PLD gene, with a sequence identical to the liver cDNA, is present in the mouse genome. The main sources of GPI-PLD are liver and brain, although RT-PCR showed expression in macrophage and pancreatic cell lines, suggesting that most cells express GPI-PLD. High GPI-PLD expression levels in liver were detected in CBA/Ca and diabetic-NOD mice, whilst obese (ob/ob) and insulin-resistant (db/db) mice showed relatively lower levels of expression. These results suggest a possible role for insulin in the regulation of GPI-PLD expression.

Co-transfection of GPI-PLD and placental alkaline phosphatase (PLAP) resulted in cleavage of PLAP from the cell surface. Co-expression of anti-sense GPI-PLD demonstrated that PLAP cleavage was catalysed by transfected GPI-PLD. However, no evidence of endogenous GPI-PLD activity on endogenous GPI-anchored proteins was obtained.

To study the cell-specific localisation of GPI-PLD expression plasmids encoding GPI-PLD fused to different tags (GFP, Flag and Myc) were constructed. A significant amount of GPI-PLD produced following transfection of these plasmids into mammalian cell lines remains cell associated. A combination of sucrose gradients subcellular fractionation and microscopy analysis suggest that GPI-PLD is associated with a Golgi-related compartment. No association with caveolae was observed even following co-expression with wild type or mutant forms of caveolin-3. These results support the hypothesis of an intracellular site of action for GPI-PLD.
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<tr>
<td>Amp</td>
<td>Amperes</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAP</td>
<td>Contig assembly program</td>
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<tr>
<td>Cav</td>
<td>Caveolin</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>Co</td>
<td>Coenzyme</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine mono-phosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CIAP</td>
<td>Calf intestine alkaline phosphatase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Minimum essential medium Eagle</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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EST  Expressed sequence tags
FCS  Foetal calf serum
FITC Fluorescein isothiocyanate
GFP  Green fluorescent protein
GHGP German Human Genome Project
GPI  Glycosylphosphatidylinositol
GPI-PLD GPI-phospholipase D
GPS  Genome Priming System
HA  Hemagglutinin
HEPES N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP Horse radish peroxidase
ICS Intracellular staining
IPG  Inositolphosphoglycan
IPTG Isopropyl β-D-thiogalactopyranoside
LPA  Lysophosphatidic acid
LPS  Lipopolysaccharide
MES  2-(N-Morpholino)-ethanesulfonic acid
M-MLV RT Moloney Murine Leukemia Virus reverse transcriptase
MOPS 3-(N-morpholino)-propane sulphonic acid
NCBI National Center for Biotechnology Information
NP-40 Nonidet P-40
nt  Nucleotides
PA  Phosphatidic acid
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PC  Phosphatidylcholine
PCR Polymerase Chain Reaction
PEG Polyethylene glycol
PFA Paraformaldehyde
PH Pleckstrin homology
PIVES 1,4 Piperazine bis (2-ethanosulfonic acid)
PL Phospholipase
pmol picomoles
RNA Ribonucleic acid
RPA Ribonuclease protection assay
SDS Sodium dodecyl sulphate
TBS Tris buffered saline
TBS-T TBS-Tween 20
TEMED N,N,N',N'-tetramethyl-ethylenediamine
TGN Trans-Golgi network
TID Type I diabetes
TLCK Nα-p-Tosyl-L-Lysine chloromethyl ketone hydrochloride
TNFα Tumor necrosis factor alpha
TNs Transposase
VSG Variant surface glycoprotein
X-gal 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
CHAPTER 1
GENERAL INTRODUCTION

The work presented in this thesis relates to the characterisation of an enzyme, glycosylphosphatidylinositol-phospholipase D (GPI-PLD) that is presumed to regulate the presence of a special class of membrane proteins, the GPI-anchored proteins on the cell surface. The main topics which are relevant are outlined in this introduction. These are a) biology of GPI-anchored proteins, b) phospholipases, with special emphasis on GPI-PLD as potential regulator of GPI-anchored protein release, and c) specialised membrane domains known as caveolae and their major component, caveolin. Within the context of this information, the aims of this thesis will be described.

1.1 MEMBRANE PROTEINS

1.1.1 Association of proteins with plasma membrane

Membranes are composed of lipids and associated proteins. Lipids are amphipathic molecules in that they contain a polar head group and hydrophobic tails. When placed in an aqueous environment, lipids spontaneously associate to form sealed bilayers with the hydrophilic head groups of lipids exposed to water. Lipids provide the basic structure of biological membranes and the specific functions are carried out by proteins. The amount and types of proteins in cell membranes are highly variable, however the usual plasma membrane contains about 50% of its mass as protein (Alberts et al., 1994).

Proteins interact with the lipid bilayer in several different ways (See Figure 1.1). To associate with membranes some proteins have hydrophobic domain(s), generally containing $\alpha$-helices. These structures which can form all their
backbone hydrogen bonds internally, in a predominantly regular arrangement, are stable within the hydrophobic membrane environment (Stevens and Arkin, 1999).

Transmembrane proteins, as membrane lipids, are amphipathic and their hydrophobic domains, typically an α-helix composed of amino acids with hydrophobic side-chains, pass through the bilayer, interact with the hydrophobic tails of the lipids and anchor the protein to the bilayer. The hydrophobicity of some of these proteins is increased by the covalent attachment of a fatty acid chain that is inserted in the cytoplasmic leaflet of the lipid bilayer. Some other transmembrane proteins have multiple membrane spanning domains (examples 1 and 2 in Figure 1.1) (Alberts et al., 1994).

**Figure 1.1.** Association of proteins with the lipid bilayer. Most transmembrane proteins extend across the lipid bilayer as single α-helix (1) or as a multiple α-helices (2). Some of these proteins have a covalently attached fatty acid chain inserted in the cytoplasmic monolayer (1). Other membrane proteins which are located on the internal side of the membranes are attached to the lipid bilayer via fatty acids or lipids (3). Some proteins are attached to the internal (4) or external face (5) of the membrane by non-covalent interactions with other transmembrane proteins. A special type of integral membrane proteins (6), associate with the external face of the lipid bilayer through a small lipidic molecule known as glycosylphosphatidylinositol (GPI). Drawing taken from Alberts et al., 1994.

Other membrane proteins do not extend across the lipid bilayer and are located in the internal side of membranes. These proteins are anchored to the lipid bilayer via fatty acids or lipid chains called prenyl groups that are covalently attached to the protein (example 3, Figure 1.1).
CHAPTER 1. GENERAL INTRODUCTION.

Some proteins do not extend into the hydrophobic interior of the membranes at all and are attached to the internal (example 4, Figure 1.1) or external face (example 5, Figure 1.1) of the membrane by non-covalent interactions with other transmembrane proteins. Based in these different forms of interaction, membrane proteins can be viewed as either integral or peripheral. Integral membrane proteins include the transmembrane proteins and lipid anchored proteins. Peripheral membrane proteins are non-covalently associated with other membrane proteins (Stevens and Arkin, 1999).

One special group of integral membrane proteins is that of proteins anchored to the lipid bilayer by a relatively small lipid molecule, the glycosylphosphatidylinositol (GPI). The GPI anchor is linked to the protein by replacement of the transmembrane segment soon after its translation in the endoplasmic reticulum (example 6, Figure 1.1). The structure and function of GPI-anchored proteins will be discussed in more detail in the next section.

1.1.2 GPI-anchored proteins in mammals

In recent years, many different proteins of eukaryotic cells anchored to membranes by covalent linkage to GPI have been described. These proteins lack a transmembrane domain, have no cytoplasmic tail and are located on the extracellular side of the plasma membrane (Cross, 1990). The GPI anchors are widely distributed in eukaryotes and so far, over 200 GPI-anchored proteins have been described (Ferguson and Williams, 1988b; Kasahara and Sanai, 2000). Animal groups where GPI-anchored proteins have been described include chickens (Henke et al., 1996), fish rays (Bucht and Hialmarsson, 1996), sea urchin (Ohta et al., 2000), fruit flies (Incardona and Rosenberry, 1996), grasshopper (Chang et al., 1992), fungi (Bruneau et al., 2001), and protozoa (trypanosomes, leishmanii, paramecium and plasmodium) (Ferguson and Williams, 1988a; McConville and Ferguson, 1993; Schofield et al., 1994; Tachado et al., 1997).
The basic structure of GPI anchors has been determined and despite the diversity of eukaryotic organisms in which they are found, GPI anchors share a common core structure. In mammalian cells the conserved common structure is constituted of a lipidic portion that can be an alkyl-acyl glycerol, diacylglycerol (DAG) or ceramide. All GPIs also contain a phosphorylated ‘6-sided’ myo-inositol glycosidically linked to nonacetylated glucosamine (GlcN), which in turn is coupled to one oligosaccharide. The presence of nonacetylated GlcN is characteristic of GPI anchors (Homans et al., 1988). The oligosaccharide portion of the molecule often contains three mannose residues (Figure 1.2) and the diversity of GPI molecules is reflected in the nature of the different branching groups of the glycan (Stevens, 1995). Other variations in the GPI-structures are found in the linkages used for the attachment of the hydrocarbon chains (ester vs. ether), the number, length and the degree of saturation of the hydrocarbons chains. In addition, the myo-inositol ring may be modified by an acyl chain. Some of these differences appear to be species-specific (Ferguson, 1999; McConville and Fergusson, 1993).

GPI-anchored proteins form a highly diverse family of molecules that includes membrane-associated enzymes, growth factor receptors, adhesion molecules, activation antigens, differentiation markers, complement molecules and protozoan coat components (Ferguson and Williams, 1988b). See Table 1.1. Although GPI anchors can be considered primarily as an alternative to hydrophobic transmembrane polypeptide anchors, their presence confers functional characteristics such as low turn over rates, increased lateral mobility and, sorting to the apical surface of polarised cells (Horejsi et al., 1999). A GPI anchor may also allow a protein to associate in cholesterol and glycosphingolipid-rich lipid rafts, to be selectively released from the cell-surface by the action of phospholipases and simultaneously to act as substrates for the generation of inositol phosphoglycans (IPG) second messengers (Hanada et al., 1995; Sargiacomo et al., 1993).
GPI-anchored proteins can be released from membranes with specific phospholipases and can be recovered from the detergent-insoluble pellet after Triton X-114 treatment of membranes. All GPI-anchored proteins are initially synthesised with a transmembrane anchor, but after translocation across the membrane of the endoplasmic reticulum, the ecto-domain of the protein is cleaved and covalently linked to a preformed GPI anchor by a specific transamidase enzyme (Ferguson and Williams, 1988b).
Table 1.1. Functional activity of GPI-anchored proteins. Some of the GPI-anchored proteins reported in mammalian tissues and their functions.

### 1.1.3 Biosynthesis of GPI anchors

The synthesis of GPI anchors has been extensively studied in cell-free systems of *Trypanosome brucei* and since the core structure of all GPI anchors is highly conserved, it is believed that the synthetic pathway is also highly conserved among different organisms (Doering et al., 1989; Masterson et al., 1989; Menon et al., 1990).

Despite some differences with respect to specific modifications after the core structure assembly (Gerold et al., 1996), studies in different organisms, from protozoans to higher eukaryotes, have demonstrated a high similarity in the biosynthetic pathways of GPI anchors (Ferguson, 1999; McConville and Ferguson, 1993; Tomavo et al., 1992). The GPI anchors are preassembled by sequential addition of glycan components to
phosphatidylinositol (PI) before being transferred to a nascent polypeptide. The initial step in the biosynthesis of GPI anchors consists in the transfer of N-acetyl-glucosamine from UDP-N-acetyl-glucosamine to phosphatidylinositol (see Figure 1.3). The product of this reaction, N-acetyl-glucosamine-phosphatidylinositol, is then deacetylated to form glucosamine-phosphatidylinositol (Doering et al., 1989; Gerold et al., 1999). Studies on mutant cells of GPI-biosynthesis indicate that the first step of the pathway requires a complex of four associated proteins which are the products of PIG-A, PIG-C, PIG-H and GPU genes (Hirose et al., 1991; Stevens and Raetz, 1991; Watanabe et al., 1988). Once glucosamine-phosphatidylinositol is synthesised, three mannose residues are subsequently added through a sequence of reactions involving the transfer of activated mannose from the hydrophobic donor, dolichol-phosphate mannose (DeGasperi et al., 1990). The product of the PIG-B gene has been identified as responsible of the addition of at least the third mannose (Takahashi et al., 1996). The subsequent addition of ethanolamine-phosphate to the third mannose by the hydrophobic donor phosphatidylethanolamine completes the biosynthesis of GPI anchors (Menon et al., 1993).

All the GPI biosynthetic intermediates have been located on the external face of microsomal vesicles, suggesting that the principal steps of GPI anchors synthesis occur in the cytoplasmic leaflet of the endoplasmic reticulum (ER) (Vidugiriene and Menon, 1994). It has been suggested, however, that a biosynthetic intermediate, possibly the phosphoethanolamine-containing anchor precursor, is translocated across the ER to the luminal leaflet where the assembly is completed. The protein(s) involved in this translocation process has not been identified so far (McConville and Menon, 2000).

The transfer of the GPI anchor to a nascent polypeptide chain occurs in the luminal side of the ER by a transamidase-type process and soon after completion of polypeptide synthesis. During this process the carboxy terminus of a polypeptide is
Figure 1.3. Biosynthesis of GPI anchors. Synthesis of GPI anchors occurs in the cytoplasmic side of the endoplasmic reticulum (ER). In the initial step, N-acetyl glucosamine is transferred to the phosphatidylinositol by a complex of four proteins (encoded by PIG-A, PIG-C, PIG-H, GPI 1 genes). Subsequently, three mannose residues are added in reactions catalysed by the product of gene PIG-B. After addition of the third mannose residue, ethanolaminephosphate is added and it is believed that this biosynthetic intermediate is translocated by a transamidase complex to the lumen of the ER, where the transfer of the GPI anchor to a polypeptide takes place. Adapted from Ferguson, 1999.
cleaved off and replaced by the GPI anchor via the amino function of the terminal ethanolamine-phosphate (Spurway et al., 2001). Comparison of DNA sequences of different GPI-anchored proteins demonstrated that the carboxy end in these proteins does not have a specific motif-like sequence but a hydrophobic region and a stretch of three consecutive amino acids 10-12 residues amino-terminal to the hydrophobic region (Udenfriend and Kodukula, 1995).

1.1.4 Free GPI

The cleavage of membrane lipids and generation of lipid-derived second messengers such as IP₃, DAG, and ceramide has been extensively studied in many different biological systems (Divecha et al., 1995). In the past years, however, the hydrolysis of GPI's has been described as an alternative pathway for receptor-mediated signal transduction.

Although the free GPI molecules have some characteristics in common with GPI anchors, their structure is less well defined. Several studies suggest that free GPI molecules contain phosphatidylinositol linked to non-N-acetylated glucosamine. Saturated fatty acids such as palmitate and myristate have also been identified (Gaulton and Pratt, 1994). The free GPI are hydrolysed to biologically active molecules called inositolphosphoglycans (IPGs). Two main types of IPG have been described: the A-type contains a variable lipidic portion, phosphorylated myo-inositol, glucosamine, and galactose; whereas the P-type contains a lipidic portion, phosphorylated chiro-inositol, and galactosamine which is linked to mannose residues. One special feature of IPGs is that they mimic some of the short- and long-term effects of insulin (Mato et al., 1987; Merida et al., 1988; Pak and Lamer, 1992).
IPG A- and P-type regulate the activity of many different proteins involved in glucose and lipid metabolism. The A-type activates acetyl-CoA carboxylase, and phosphodiesterases. It inhibits the activity of cAMP-dependent protein kinases and adenylate cyclase and cAMP phosphodiesterases. IPG P-type activates pyruvate DH phosphatase and glycogen synthase. The IPG P-type also inhibits cAMP-dependent protein kinases and glucose-6 phosphatase (Rademacher et al., 1994; Romero et al., 1993; Varela-Nieto et al., 1996). See Figure 1.4.

The generation of IPGs is modulated by a variety of agonists whose membrane receptors fall into three categories: receptors with intrinsic tyrosine kinase activity, receptors associated with tyrosine kinases, and receptors with seven transmembrane domains (reviewed in Varela et al., 1996). Studies on the insulin-signalling pathway have led to suggest that the cleavage of GPI molecules by a GPI-specific phospholipase could be responsible for the release of IPG molecules (Saltiel and Cuatrecasas, 1986; Suzuki et al., 1991; Jones et al., 1997). Figure 1.4 outlines schematically a model suggesting a role of phospholipases with specificity for GPI molecules in the generation of IPGs. It should be noted that such a model is controversial, and that multiple questions have been raised. Firstly, the identity of the precursors of IPGs is not known, and whether GPI anchors or free GPIs are the potential precursors of IPG mediators, has been extensively debated. Cleavage of GPI anchors after stimulation by different agents is considered as indirect evidence in support of the hypothesis that activation of phospholipase(s) could liberate IPGs (1).

One problem with the GPI anchors as precursors of IPGs is that the cleavage mechanism would also necessitate the action of a protease to cleave the inositol-glycoprotein generated after the phospholipase cleavage (discussed by Varela-Nieto, et al, 1996).
However, the analysis of the composition of GPI molecules has shown that “free-GPI” molecules lack ethanolamine, which is an essential component of the GPI anchors (Mato et al., 1987). This combined with the observation that T lymphocytes with an impaired synthesis of GPI-anchored proteins are able to synthesise an insulin-sensitive GPI suggest that free-GPI, and not the GPI anchors, are - or at least can be- the precursors of IPG second messengers (Avila et al., 1992).

**Figure 1.4.** Suggested model for phospholipases and their role in the generation of IPG second messenger molecules. In this model, the activation of the insulin receptor (IR) by insulin results in the activation of a phospholipase that cleaves either GPI anchors or free GPs (1) to generate IPG. The IPGs extracellularly generated are then transported into the cell and supporting the extracellular generation of IPGs a transport system has been described in hepatocytes. One important question, however, is how the intracellular phospholipases could become associated with the cell membrane in such a way to cleave substrates associated with the outer leaflet of the membrane. An alternative mechanism for the generation of IPGs could involve the cleavage of GPI molecules distributed in intracellular membrane compartments (2) and caveolae (3). The identity and the cellular localisation of the specific phospholipase responsible of the cleavage of GPI molecules have not been well established. The properties of GPI-PLC and GPI-PLD as responsible of the cleavage of GPI molecules is discussed in further sections of this chapter. This model is modified from Field, 1997.
Secondly, another important issue is related to the location of the precursors of IPG molecules. Some studies have suggested that the precursors of IPGs are mainly located in the outer membrane leaflet (Alvarez et al., 1988; Varela et al., 1990). This location of the GPIs poses the question of how the specific phospholipase(s) can become activated by receptors and associated with the cell membrane in order to cleave the GPI molecules. In the particular case of insulin, it is not known whether cross-linking of the insulin receptor (IR) results in a direct activation of the GPI-specific phospholipase(s) or whether other mediators exist (Field, 1997).

Other questions relate to how extracellularly-generated IPGs later can enter the cells, and how all these processes are regulated (Varela-Nieto et al., 1996). In support of the extracellular origin of IPGs, studies using myocytes cultures have demonstrated an increase of the extracellular levels of IPGs upon stimulation with insulin. In addition, the effects of the stimulation with insulin are inhibited in the presence of anti-IPG antibodies (Romero et al., 1988, 1990), and evidence that a regulated IPG transport system exists in rat hepatocytes allowing extracellularly generated IPGs to be actively transported into the cells has been provided (Avarez et al., 1991). Alternatively, experiments have demonstrated that GPI molecules can also be located in intracellular membrane compartments, suggesting an intracellular origin for IPGs (Baumman et al., 2000). The possibility that such intracellular located IPG-precursors could give rise to the second IPG messengers would provide the basis for a less complicated model not requiring the need for transporting IPGs into the cell.

Thirdly, other important points are related to the identity and the subcellular localisation of the specific phospholipase(s) catalysing the formation of IPGs. GPI-PLC and GPI-PLD have been suggested as the enzymes responsible of the cleavage of GPI molecules. The properties of these enzymes are described in the next sections of this thesis.
1.2 PHOSPHOLIPASES

1.2.1 General characteristics of phospholipases

Lipids constitute a diverse group of slightly polar natural compounds such as free fatty acids, neutral glycerides, waxes, phospholipids, sphingolipids, oxylipids, and sterols. For a long time, lipids were considered as metabolic fuel stores and structural components of cell membranes. In recent years, however, it has been shown that lipids act as biological effectors, regulators, and mediators which participate in virtually all biological processes (Cullis et al., 1996). As second messengers, the role of membrane lipids in signal transduction mechanisms has been well established. The action of extracellular agonists results in the activation of different lipases (enzymes that catalyse the cleavage of fatty acids from the glycerol moiety of a triglyceride) to generate lipid-derived second messengers (Divecha et al., 1995). One particular group of lipases whose role in signal transduction mechanisms has been well documented is the phospholipases, which are enzymes that catalyse the hydrolysis of phosphoglycerides or glycerophosphatidates. Phospholipases and phospholipids are involved in the processes of transmitting ligand/receptor-induced signals from the plasma membrane to intracellular proteins. The primary protein affected by the activation of phospholipases is protein kinase C (PKC) which is maximally active in the presence of calcium ion and diacylglycerol (DAG) (Fukami, 2002).

Phospholipases (PL) are highly specific and based on the cleavage site in their substrates, these enzymes, are grouped into two main categories (Figure 1.5): aliphatic esterases and phosphodiesterases. The first category includes PLA₁, PLA₂ and PLB. PLA₁ hydrolyses the acyl group attached to the 1-position. This enzyme is present in all mammalian tissues and is an important virulence factor of bacteria, pathogenic fungi, and protozoa (Capper and Marshall, 2001; Chaminade et al., 1999; Hirabayashi and Shimizu, 2000).
Phospholipase A2 (PLA$_2$) constitute a complex family of enzymatic activities classified in 5 groups (I-V) depending on their localisation (secreted or cytosolic) and their regulation by Ca$^{2+}$. These enzymes have been reported in all mammalian tissues and some of them are also present in gastric secretions and venoms. PLA$_2$ type II has been studied in detail because its action can release arachidonic acid, which leads to the synthesis of prostaglandins and leukotrienes (Dennis, 1994). At least three different genes coding for PLB (also known as lysophospholipase, lecithinase B, and lysolecithinase) have been characterised in mouse and yeast. This enzyme catalyses the hydrolytic cleavage of both acylester bonds of glycerophospholipids to produce fatty acids and water-soluble glycerophosphodiesters (Merkel et al., 1999; Toyoda et al., 1999).
1.2.2 Phosphodiesterases PLC

The second group of phospholipases includes the phosphodiesterases PLC and PLD. PLC cleaves specifically phosphatidyl inositol 4,5-bisphosphate [Pi(4,5) P$_2$] and generates two second messengers, inositol-1,4,5-trisphosphate (IP$_3$), and diacylglycerol (DAG). The released IP$_3$ interacts with intracellular membrane receptors leading to an increased release of stored calcium ions. Together, the increased DAG and intracellular free calcium ion concentrations lead to increased activity of PKC (Fukami, 2002).

![Domain structure of PLC isozymes](image)

*Figure 1.6. Domain structure of PLC isozymes.* A block representation of the modular domain arrangements found in mammalian δ, β, γ and ε PLC isozymes (Williams, 1999).
So far, 11 different PLC isozymes have been identified in mammals and based on their structure and activation mechanism they are grouped into four different classes, \( \beta(1-4), \gamma(1-2), \delta(1-4) \) and \( \varepsilon(1) \) (Katan, 1998; Rhee, 2001). All members of this family share a \( \delta \)-like core sequence with a N-terminal PH domain, that allows association with the cell membrane and EF hands that contain residues which are postulated to bind \( \text{Ca}^{2+} \) ions (Figure 1.6). All PLC isoforms have also a catalytic domain, containing an \( \alpha/\gamma \) linker region that it is important for regulation of the enzymatic activity. In PLC\( \gamma \) this region has evolved to two SH2 and one SH3 domain, which are required for activation of the enzyme by tyrosine kinases. At the C-terminal end all PLC have a C2 domain that contains calcium-binding residues. PLC\( \varepsilon \) has one Ras-GFEl-like domain in the N-terminal end and two additional RA domains at the C-terminal end (Fukami, 2002; Williams, 1999).

### 1.2.3 Phospholipases C acting on GPI molecules

Phosphatidyl inositol (PI)-specific-PLC enzymes have also been isolated and cloned from *Bacillus cereus* and *Bacillus thuringiensis*. These two enzymes recognise only lipids containing the myo-inositol group and catalyse the hydrolysis of PI to generate 1,2-diacylglycerol and myo-inositol 1,2-cyclic phosphate (Griffith *et al.*, 1991). These two phospholipases show certain specificity for GPI anchors (Low, 1981; Taguchi *et al.*, 1980). In addition to the mechanisms that control their activity, differences in their substrate preference distinguish bacterial and mammalian PLCs. Bacterial PLCs do not recognise phosphorylated derivatives of PI (phosphatidylcholine or phosphatidylethanolamine) and despite having specificity for GPI molecules (Low, 1981; Taguchi *et al.*, 1980), they are unable to cleave GPI-anchored proteins with fatty acids (usually palmitate) attached to the inositol ring (Roberts *et al.*, 1988). In addition to the functional differences, no structural homology has been found after comparison.
of cDNAs coding for mammalian and bacterial PLCs (Henner et al., 1988; Kuppe et al., 1989).

A phospholipase with enzymatic properties similar to the bacterial PI-PLC has been isolated from peanuts seeds (Butikofer & Brodbeck, 1993). This phospholipase can cleave the GPI anchors of acetylcholinesterase from bovine erythrocytes and variant surface glycoprotein from Trypanosoma brucei, to produce DAG and soluble inositol-glycoproteins. In addition, a calcium-independent GPI-specific PLC associated with intracellular membranes and with homology to PI-PLC from B. cereus, has also been characterised and cloned from Trypanosoma brucei (Fox et al., 1986; Hereld et al., 1986; Bulow et al., 1989, Kuppe et al., 1989).

No mammalian GPI-specific PLC has yet been cloned. However, phospholipases with specificity for GPI-anchors have been purified from rat liver and mouse brain membranes (Fox et al., 1987; Fouchier et al., 1990; Stieger et al., 1991). At first it was believed that the hydrolysis of GPI molecules was due to the action of a GPI-specific PLC. However, studies using the phosphatase inhibitors NaF and sodium orthovanadate, demonstrated the presence of phosphatidic (PA) in addition to diacylglycerol. These results indicated that DAG released from cleavage of GPI molecules might be not due to the action of a GPI-PLC but the action of a GPI-phospholipase D (GPI-PLD) acting in concert with a phosphatase. In these reactions, the phosphatidic acid (PA) released by the activity of GPI-PLD is subsequently dephosphorylated by a phosphatase to generate DAG (Heller et al., 1992).

There is, however, evidence to support the existence of a GPI-PLC. Firstly, the rapid generation of DAG parallels the time course of the hydrolysis of GPI and also of the generation of IPG (Suzuki et al., 1991). Secondly, by using anti-CRD antibodies (Hooper et al., 1991) that selectively recognise an epitope created only after cleavage of GPI molecules by a PLC, Vogel et al., 1992 demonstrated that soluble forms of the
GPI-anchored 5'-nucleotidase in bovine cerebral cortex and the electric organ of the electric ray are derived from membrane-bound forms through the action of a PLC (Vogel et al., 1992).

The existence of a GPI-specific PLC responsible for involved in the cleavage of GPI molecules has received more support by recent in vitro experiments showing that GPI-anchored membrane dipeptidase (MDP, also known as renal dipeptidase) cleaved from the cell surface adipocytes and porcine proximal tubules is recognised by anti-CRD antibodies (Movahedi and Hooper 1997, Park et al., 2001). Interestingly, it has been shown that the cleavage of MDP is regulated positively by insulin (Movahedi and Hooper, 1997) or negatively by nitric oxide (Park et al., 2002a). Further studies on the renal dipeptidase have also provided the first direct evidence of the cleavage of GPI-anchored proteins by GPI-PLC in vivo (Park et al., 2002b). The involvement of other phospholipase in the in vivo cleavage of membrane dipeptidases could also be possible as it has been reported that phospholipase A is also able to induce the release of this enzyme (Hooper, et al., 1997). The cloning of the gene coding for GPI-PLC will be important to analyse the biological roles and significance of this phospholipase.
1.2.4 Phosphodiesterases PLD

PLDs distributed in plants, bacteria, yeast, fungi, and mammals, catalyse the hydrolysis of phosphatidylcholine (PC) and other phospholipids to generate phosphatidic acid (PA). PLDs are activated in response to occupation of cell surface receptors including those of the heterotrimeric G-protein and tyrosine kinase families (Banno, 2002; Frohman and Morris, 1999; Liscovitch et al., 2000). PA may itself act as a signal molecule, by activating a PA-activated kinase, or can be hydrolyzed to form DAG and lysoPA which are able to recruit or modulate other target proteins (Banno, 2002).

PLDs constitute a complex and diverse family of isozymes that have been cloned from plants (PLDα, β, and γ), yeast (SPO14/PLD1), and humans (PLD1a) (Hammond et al., 1995; Liscovitch et al., 2000; Rose et al., 1995). A splice variant of PLD1a (PLD1b) lacking 38 residues at the N-terminal region and another PLD with ~50% homology to PLD1a have also been described (Colley et al., 1997; Hammond et al., 1997). Other genes cloned from fungus, bacteria and nematodes complete all the PLD members reported so far (Banno, 2002; Kanoh et al., 1998; Liscovitch et al., 2000). See Figure 1.7.

Structurally, all the PLD family members share four core domains I-IV (in red in Figure 1.7) and additional domains (in blue) are conserved in the yeast, human and nematode sequences, but are absent from the plant and the bacterial sequences. Domains II and IV contain a short sequence motif termed HKD or phosphatidyl transferase motif. The HKD motifs are required for the catalytic activity and may dimerise to form an active centre. The conservation of these domains among all the members of the PLD family confers a similar mechanism of action. Two PX (in green in Figure 1.7) domains in the N-terminal region, present only in mammals and nematodes, are important for intra- or inter-molecular interactions. Mammals, nematodes and yeast share two pleckstrin homology (PH) domains that are not present in plants and bacteria. PH domains lack
catalytic activity but are important for the association of PLDs with membranes and other proteins (Liscovitch et al., 2000). All eukaryotic PLDs share a conserved C-terminal motif (CT) which is not present in bacteria (in light blue in Figure 1.7). As a distinctive characteristic, plant PLDs have a C2 domain (in yellow in Figure 1.7) in the N-terminal region which is believed to be involved in binding acidic phospholipids in a Ca\(^{2+}\)-dependent manner (Banno, 2002; Liscovitch et al., 2000).

Additional eukaryotic PLDs activities including the oleate-activated PLD, the yeast Ca\(^{2+}\)-dependent phosphatidylserine (PS)/phosphatidyl ethanolamine (PE)-hydrolysing PLD, mitochondrial PE-PLD, N-acyl-PE-PLD, Lyso-PLD, PI-PLD, and glycosylphosphatidylinositol (GPI)-PLD have also been described (Cockcroft, 1984; Liscovitch et al., 2000). Because of the interests in this work, GPI-PLD will be described in more detail in the next sections.
1.3 GLYCOSYLPHOSPHATIDYLINOSITOL PHOSPHOLIPASE D (GPI-PLD)

1.3.1 GPI-PLD enzymatic activity

Different phospholipases hydrolyse GPI-anchored proteins, GPI-lipids, and related molecules. These anchor-degrading activities were originally suggested to be due to the action of an inositol phospholipid-specific phospholipase C (Griffith et al., 1991; Varela-Nieto et al., 1996). Further experiments demonstrated that a specific GPI-phospholipase D (GPI-PLD) hydrolyses the inositol phosphate linkage of GPI-anchored proteins and free-GPI to generate soluble inositol-glycoproteins, phosphatidic acid and IPGs (Figure 1.8). So far, GPI-PLD is the only highly specific-GPI hydrolysing enzyme that has been described in mammals (Metz et al., 1994).

The minimal structural motif recognised by GPI-PLD is glucosamine-phosphatidylinositol, which has been identified as the core of free GPI molecules. In contrast to other phospholipases, GPI-PLD is highly specific and does not cleave other phospholipids such as phosphatidylethanolamine, phosphatidylcholine or phosphatidyl inositol (Davitz et al., 1987; Deeg and Davitz, 1995).

GPI-PLD was originally detected as an enzyme capable of removing the anchor of alkaline phosphatase during its extraction from pig kidney microsomes (Low and Zilversmit, 1980). A few years later, it was detected in human serum (Davitz et al., 1987) and because of its relatively high (~5-10 µg/ml) concentration (Davitz, 1989; Huang et al., 1990), it was considered responsible for the release of GPI-anchored proteins (Low and Prasad, 1988).

In support of this, soluble forms of GPI-anchored proteins such as axonin-1, ADP-ribosyl transferase, Thy-1, decay accelerating factor (DAF), N-CAM, 5'-nucleotidase, CD14, and urokinase receptor have been detected. These soluble forms of GPI-
anchored proteins are likely to be released by a regulated hydrolysis of their GPI anchor (Almqvist and Carlsson, 1988; Delgado et al., 1999; Gennarini et al., 1984; Klemens et al., 1990; Lieberheimer et al., 1997; Medof et al., 1987; Metz et al., 1994; Nemoto et al., 1996; Wilhelm et al., 1999).

In addition, GPI-PLD can cleave free GPIs, with the subsequent production of inositol-phosphoglycans (IPGs), which are known to act as second messengers in the signal transduction pathways of insulin and other hormones and cytokines (Figure 1.4). This activity has led to the theory that GPI-PLD could be a member of the group of phospholipases implicated in the control of signal transduction events (Jones et al., 1997; Jones and Varela-Nieto, 1998; Rademacher et al., 1994).
Despite its potential regulatory roles, the mechanisms that activate and induce GPI-PLD activity, and the actual site where it exerts its activity are not well established. GPI-PLD activity has been only demonstrated in vitro in the presence of detergents or cholesterol-binding agents. A direct demonstration of the cleavage of cell-associated GPI-anchored proteins on living cells by GPI-PLD has not been demonstrated so far, and therefore conclusive evidence for the physiological role of GPI-PLD is still lacking, suggesting that its function may be highly regulated (Bergman et al., 1994; Davitz, 1989; Low and Huang, 1991).

1.3.2 Gene structure

GPI-PLD was first cloned from a bovine liver cDNA library using degenerate oligonucleotide probes based on the amino acid sequences of fragments obtained after trypsin treatment of bovine serum GPI-PLD (Scallon et al., 1991). Subsequently, two human GPI-PLD cDNAs were isolated from liver and pancreas libraries (Tsang et al., 1992). Most recently, a mouse cDNA isolated from a mouse glucagonoma (pancreas) cell line has been reported (LeBoeuf et al., 1998). The GPI-PLD genes in these three species contain open reading frames of 2520, 2523, and 2511 bp, respectively. These genes also include 3' untranslated regions which contain stop codons and polyadenylation sites (Huang et al., 1990; LeBoeuf et al., 1998; Scallon et al., 1991; Schofield and Rademacher, 2000; Tsang et al., 1992).

The analysis and screening of human genomic DNA libraries allowed elucidation of the complete structure of the GPI-PLD gene which encodes the previously described pancreatic-derived cDNA (Schofield and Rademacher, 2000; Tsang et al., 1992). The human GPI-PLD gene has characteristics typical of a vertebrate gene, encompassing 25 exons (Figure 1.9). Exons 1-24 are small, ranging from 27 to 208 bp, whereas the final exon is 3224 bp. The DNA sequences coding for the complete gene have been
deposited in the Sanger Research Centre database (accession number AL031230) and the EMBL database (accession numbers AJ400872-AJ400876) (Schofield and Rademacher, 2000). A search for motifs in the human GPI-PLD gene revealed several weak promoter motifs, a transcription start site in the 5' untranslated region, and two consensus polyadenylation signals in the final exon (3' end). FISH analysis has shown that human GPI-PLD maps to chromosome 6p22.1, whilst the mouse gene is located in the proximal region of chromosome 13 (LeBoeuf et al., 1998; Schofield and Rademacher, 2000).

1.3.3 GPI-PLD protein characteristics.

The bovine, human and mouse cDNA sequences predict polypeptide chains of 840, 841 and 837 amino acids, respectively. The three sequences include a 23-amino acid signal peptide and show 79% homology (LeBoeuf et al., 1998; Scallon et al., 1991; Tsang et al., 1992). The mature proteins have a calculated mass of ~90 kDa, but as GPI-PLD is synthesised as a glycosylated protein and because of alternative oligosaccharide processing in different cell types, mature forms of 100-110 kDa have been detected. GPI-PLD is an amphiphilic protein that in serum is associated with high density lipoproteins (HDL). Immunoprecipitation studies of GPI-PLD have shown that it is mainly associated with apolipoprotein (apo) A-I and apoA-IV in the form of small (8 nm) particles/complexes (Deeg et al., 2001a; Hoener et al., 1993). The lack of activity of GPI-PLD on intact membranes suggests that GPI-PLD is catalytically inactive in serum or require changes in the membrane environment of the substrate to allow cleavage (Bergman et al., 1994; Low and Huang, 1991). apoA-I stimulates GPI-PLD activity in vitro (Hoener et al., 1993) but it is not clear whether apo A-I is sufficient to stimulate GPI-PLD-mediated cleavage of cell surface-bound GPI-anchored proteins. Hence, the function of GPI-PLD in serum and why it associates with HDL are unknown (Deeg et al., 2001a).
Figure 1.9. Structure of the human GPI-PLD gene. The human GPI-PLD encompasses 25 exons. Exons 1 to 24 are small, ranging from 27 to 208 bp, whereas the final exon 25 is 3224 bp. The DNA sequences coding for the complete gene have been deposited in the Sanger Research Centre database (accession number ALO31230) and the EMBL database (accession numbers AJ400872-AJ400876). Drawing based on the information by Schofield and Rademacher, 2002.)
A search for motifs within the mouse protein sequence reveals two potential amidation sites, one cAMP phosphorylation site, and one tyrosine phosphorylation site. Multiple sites for casein kinase II phosphorylation, protein kinase C phosphorylation and asparagine glycosylation were also found (LeBoeuf et al., 1998). Furthermore, it has been shown that GPI-PLD is phosphorylated in vitro by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and by tyrosine kinase. Phosphorylation by PKA resulted in a significant decrease of the enzymatic activity (Civenni et al., 1999).

Analysis of the deduced amino acid sequence reveals four regions of internal homology with a high similarity to metal-ion binding domains in the α-subunits of proteins belonging to the family of integrins (Scallon et al., 1991; Springer, 1997). These regions contain sequences with some similarity to the E-F hand (responsible for metal ion binding) found in a number of Mg\(^{2+}\) and Ca\(^{2+}\)-binding proteins such as parvalbumin, troponin c, and calmodulin (Huang et al., 1990; Hynes, 1992). Metal analysis have indeed demonstrated the presence in GPI-PLD of 5 and 10 atoms/molecule of Ca\(^{2+}\) and Zn\(^{2+}\), respectively (Li et al., 1994).

Since removal of Ca\(^{2+}\) by divalent cation chelators, had no effect on the enzymatic activity, it has been proposed that the metal-ion binding sites in GPI-PLD have a structural or regulatory role but are not required for GPI hydrolysis (Li et al., 1994). Further studies where the predicted Ca\(^{2+}\)-binding sites were deleted or mutated resulted in a decrease in the release of GPI-PLD suggesting a possible role in the folding, intracellular transport or secretion (Li et al., 1999).

Modelling studies with software programs of mouse GPI-PLD sequence showed the presence of α-helix areas and sites of β-turn or β-sheet structures (LeBoeuf et al., 1998) which are organised in a β-propeller domain. However, no crystallographic analysis has been reported yet. The hypothetical structure fits with a propeller structure.
in which seven sets of four anti-parallel beta-strands twist around a central axis resembling a propeller pattern in which a set of four beta sheets arranged in an anti-parallel fashion make up one propeller blade (Springer, 1997) (Figure 1.10).

Trypsin treatment of native bovine GPI-PLD results in the generation of three different fragments that are resistant to further cleavage. These three fragments with molecular weights ranging between 30-45 kDa remain associated and retain the enzymatic activity. Although each fragment is predicted to contain at least two cysteine residues, they are not involved in the formation of disulfide bonds. Trypsin treatment suggests that the protein is organized in three distinct and separable domains (Li et al., 1994).

Based on these studies it has been suggested that the active site of GPI-PLD may reside in the N-terminal region, within the first 275 amino acids (Heller et al., 1994). The C-terminal end, however, is also important for the enzymatic activity. Either the elimination of two to five C-terminal amino acids or point mutations involving a tyrosine within this region resulted in a decreased or total inhibition of GPI-PLD activity. From this study it has been suggested that mutated tyrosine is part of a phosphorylation motif important for the activity of GPI-PLD or that this tyrosine is important for maintaining the β -propeller structure and this is reflected in the enzymatic activity (Stadelmann et al., 1997).

In addition, it has been shown that GPI-PLD activity is inhibited by lipid A, phosphatidic acid and lysophosphatidic acid (LPA). It has been proposed that the mechanism involves the binding of these inhibitors to a central hydrophobic cavity predicted by the β-propeller model for integrin α -subunits and GPI-PLD (Low and Stutz, 1999). The detection of micromolar concentrations of LPA in serum suggests a possible mechanism for regulation of GPI-PLD activity (Moolenaar, 1995; Tgyi and Miledi, 1992).
Figure 1.10. \( \beta \)-propeller domain structure of integrins. Ribbon diagram of the model for the integrin \( \alpha 4 \)-subunit \( \beta \)-propeller domain is shown in this picture. From the common convention for \( \beta \)-propellers, \( W \) indicates each of the seven \( \beta \)-sheets (in different color) present in the molecule. A hypothetical polypeptide finger in the central cavity is grey. Cysteine residues in disulphides are black. Ca\( ^{2+} \) ions and a hypothetical Mg\( ^{2+} \) ion are gold and silver spheres, respectively (Springer, 1997).

1.3.4 GPI-PLD expression

Despite GPI-PLD being relatively abundant in serum, for some time it was not clear which cells or organ(s) were the source of this enzyme. It is considered now that liver is the major source of GPI-PLD (LeBoeuf et al., 1998; Schofield and Rademacher, 2000), and supporting this, it has been demonstrated that its concentration is decreased in the serum of liver-damaged patients (Maguire and Gossner, 1995; Raymond et al., 1994; Tujioka et al., 1998).
There is evidence, however, indicating that cells of the inflammatory response and different organs such as adrenal gland, lung, brain and pancreas may also contribute to the circulating GPI-PLD (Deeg and Verchere, 1997; Hoener et al., 1990; Metz et al., 1991; Rhode et al., 1999; Stadelmann et al., 1993). It has been shown that a number of different cellular types express, and some of them secrete, GPI-PLD. Human and mouse myeloid cell lines, mast cells, pancreatic islet α and β cells, keratinocytes, sensory neurones, and hepatocytes are some examples of cells in which GPI-PLD has been detected. In some cases, GPI-PLD detection has been based on the use of antibodies or enzymatic activity. Because of the high concentration in serum, the results obtained by these approaches are not absolutely reliable as contamination can account for the GPI-PLD detected. In view of this, evaluation of mRNA levels has been used to detect GPI-PLD (Deeg and Verchere, 1997; Lieberheimer et al., 1997; Metz et al., 1991; Solter and Hoffmann, 1999; Xie and Low, 1994).

1.3.5 GPI-PLD and diseases

GPI-PLD localisation in atherosclerotic coronary arteries has led to the suggestion that this enzyme participates in the pathogenesis of this disease (O’Brien et al., 1999). Some evidence also suggests that GPI-PLD may be associated with diabetes and insulin metabolism. First, it has been demonstrated that IPGs, potential products of the GPI-PLD enzymatic activity, have a second messenger activity in the insulin signal transduction pathways. By regulating the activity of many different molecules (Figure 1.4), IPGs control the metabolism of glucose and lipids (Rademacher et al., 1994; Romero et al., 1993; Varela-Nieto et al., 1996). Second, the release of GPI-anchored proteins in response to insulin has been observed in different cell types (Chan et al., 1988; Lisanti et al., 1989), and the GPI-PLD-mediated generation of IPGs has been reported (Jones et al., 1997). Finally, it has been observed that GPI-PLD is produced in the pancreas and its expression regulated by insulin in isolated pancreatic cells (Deeg
and Verchere, 1997; Metz et al., 1991). Taken together all these observations suggest that GPI-PLD could be involved in the pathogenesis of diabetes.

Insulin-dependent type 1 diabetes (TID), is an organ-specific autoimmune disease resulting from the selective destruction of insulin-producing beta cells in the pancreas (Bottazzo et al., 1974; MacCuish et al., 1974). Although the mechanisms that result in the onset of the disease are not well established, the clinical manifestations have been characterised and the use of various diabetic mouse models that develop a similar type of diabetes as humans has been very helpful in understanding the disease.

One of best known mouse models of diabetes is the nonobese diabetic (NOD) mouse in which diabetes has an autoimmune aetiology that is heavily influenced by both genetics and environment. Diabetes onset in mice occurs commonly from 10-12 weeks of age and leads to hyperglycaemia, glycosuria, polyuria, ketonuria, and hypoinsulinemia with death resulting if mice do not receive insulin. As in humans, the disease involves considerable changes in lipoprotein metabolism, including changes in the expression of many apolipoproteins and in the levels of lipoprotein fractions. The different observations that relate insulin metabolism and GPI-PLD therefore suggest a possible role for GPI-PLD in the diabetes (Deeg et al., 2001b).
1.4 LIPID RAFTS

1.4.1 Structure and composition of lipid rafts

For a long time the plasma membrane was visualised as a neutral two-dimensional "fluid mosaic" solvent. In this model, of a liquid-crystalline phase, the proteins are considered to be uniformly distributed in the lipid solvent (Singer and Nicholson, 1972). This phase behaviour is true for membranes rich in phospholipids, which have low acyl chain melting temperatures. During the past years, however, it has been found that certain cell membranes are rich in sphingolipids and sterols, which have elevated acyl chain melting temperatures (Sīlvius et al., 1996). The interaction between these lipidic molecules generates different phases, including gel, liquid-ordered and liquid-disordered within the membrane. It is believed that these interaction are responsible for the formation of lipid islands or rafts enriched in glycosphingolipids, sphingomyelin and cholesterol (Brown and London, 1988).

Characterisation of the lipid rafts has been controversial. Because the original procedures to isolate these membrane structures rely on the use of detergents, there were concerns that these lipid domains formed as a result of the detergent-treatment. It could be also possible that some raft components were missing or incorporated because of the detergent and extraction conditions. The development of centrifugation/flotation methods based on high lipid content (hence low density) of lipid rafts, and the use of immuno- and electron-microscopy has been very helpful to identify structures and/or specific components associated with lipid rafts (Hooper, 1999; Simons and Toomre, 2000).

So far, it has been established that lipid rafts are enriched in glycosphingolipids, sphingomyelin and cholesterol. This particular composition allows lipid rafts to include or exclude certain proteins. Proteins with affinity for rafts include GPI-anchored proteins, doubly acylated proteins (Src-family kinases or α-subunit of heterotrimeric G
proteins), and palmitoylated transmembrane proteins (Brown and London, 1988; Simons and Toomre, 2000). Lipid rafts are localised mainly at the plasma membrane but they can also form within internal membrane compartments, such as the Golgi complex (Gkantiragas et al., 2001).

1.4.2 Caveolae

Caveolae, also know as plasmalemmal vesicles are small (50-100 nm) flask-shaped invaginations of the plasma membrane (Kurzchalia et al., 1992; Rothberg et al., 1992) (Figures 1.11A and B). These organelles were first described almost 50 years ago, but it is just in the past years that their function is finally being understood (Palade, 1953; Yamada, 1955). Caveolae have been described as a specialized form of lipid raft (Simons and Ikonen, 1997) and detected in almost all cell types, although they are particularly abundant in terminally differentiated cells such as endothelia, smooth muscle cells, adipocytes, and epithelial cells (Anderson, 1993).

In a similar way to lipid rafts, caveolae are enriched in glycosphingolipids, particularly the ganglioside GM1, sphingomyelin, and cholesterol, but they are almost depleted of phospholipids (Parton, 1994). Cholesterol is very important for the overall structure and function of caveolae. Exposure to cholesterol-binding/depleting drugs, cause caveolae and lipid rafts to dissociate, caveolin to re-localise to the Golgi/endoplasmic reticulum and GPI anchored proteins to disperse (Fielding and Fielding, 2001). GPI-anchored proteins and proteins that contain covalently linked lipids such as myristate and/or palmitate, or an isoprenoid are enriched in caveolae (Figure 1.11C). Mutations that block these lipid modifications shift the mutant protein to a membrane fraction different to caveolae (Brown and London, 1997, 1998; Ying et al., 1992).

One distinctive characteristic of caveolae is their enrichment for a 22-kDa membrane phosphoprotein, caveolin, which is so far the best biochemical marker of these membrane structures (Schlegel and Lisanti, 2001).
Figure 1.11. Structure of caveolae. Electron microscope pictures showing the morphology of caveolae at the cell membrane. A) View from below, where it can be seen how the invaginations are directed towards the inside of the cell. B) A lateral view of caveolae. C) Schematic representation of the main components of caveolae (Rothberg et al., 1992).
1.4.3 Functions of caveolae

It has been proposed that caveolae are involved in the following cell processes:

- **Potocytosis**, a form of endocytosis that does not use clathrin-coated pits. This form of internalisation starts with the attachment of small molecules to GPI-anchored receptors on the plasma membrane. The complexes ligand/receptor are clustered within caveolae and then internalised by invagination. Ligands are released from their receptors by acidification and because of their high concentration induces their diffusion into the cytoplasm (Travis, 1993).

- **Transcytosis**, the transport of macromolecules across a cell, consisting of endocytosis of a macromolecule at one side of a monolayer and exocytosis at the other side (Schnitzer et al., 1995).

- **Protein sorting**, the observation that basolateral and apical surfaces in polarised epithelial cells have a distinct protein composition, indicates that a sorting process exists. The high concentration of glycosphingolipids in the apical surface and the fact that the intracellular transport of GPI-anchored proteins depends on sphingolipids and cholesterol suggest that caveolae may be involved in the specific transport of proteins to specific cell surfaces (Muniz and Riezman, 2000). It has also been suggested that acylated proteins are targeted to the specific membrane domains by the same mechanisms as GPI-anchored proteins (Brown and London, 1997). It has been recently reported that cholesterol internalised by clathrin-coated vesicles moves from these structures to the trans-Golgi and then into membrane fractions rich in caveolin. This observation suggest that caveolae are also important in cholesterol trafficking (Fielding and Fielding, 1996; Smart et al., 1994).

- **Cell signalling**: It has been established that the nature of caveolae components and the structural features of caveolin allow the concentration of multiple signalling molecules in these organelles. It has also been shown that caveolin regulates the activity of the signal transducers concentrated in caveolae (Okamoto et al., 1998; Sargiacomo et al., 1993).
1.5 CAVEOLIN

1.5.1 Caveolin gene family

Caveolins are a family of ~22 kDa integral membrane proteins that constitute the major structural component of caveolae. Transfection of caveolin into cells that do not normally express this protein is sufficient to induce the formation of the flask shape microdomains (Lipardi et al., 1998).

Three homologous genes coding for caveolin (Cav) have been cloned and their products referred to as Cav-1, -2, and -3 (Figure 1.12). Cav-1 exists in two different isoforms (Cav-1α and Cav-1β) which are derived from alternate initiation sites. Translation of the α and β isoforms begins at methionine 1 and 32, respectively, resulting in an additional N-terminal sequence in Cav-1α (Engelman et al., 1998; Kurzchalia et al., 1992; Scherer et al., 1995; Tang et al., 1996). Cav-1 and Cav-2 are found predominantly in adipocytes, smooth muscle cells, and epithelia, whereas Cav-3 is found predominantly in muscle cell types (Tang et al., 1996). The alpha and beta isoforms of caveolin-1 display different but overlapping intracellular distribution (Dupree et al., 1993).

1.5.2 Caveolin structure

All three types of caveolin are palmitoylated, highly phosphorylated on tyrosines and have an unusual topology, very important to the interaction with other caveolin molecules. Sequence and structural analysis of Cav-1 have demonstrated the presence of a central hydrophobic domain (residues 102-134) which is believed to anchor the protein to membranes (Glenney and Soppet, 1992). This hydrophobic domain is believed to form a hairpin loop structure in the cell membrane and as a consequence, both the N-terminal (residues 1-101) and C-terminal (residues 135-178) ends face the cytoplasm (Figure 1.13).
Figure 1.12. Schematic representation of the different members of the caveolin family. Two different forms of Cav-1 have been reported (α and β) and they differ in the initiation site. The α form contains an additional N-terminal sequence of 32 amino acids. Caveolins are divided into three distinct regions: a cytoplasmic N-terminal domain (N), a hydrophobic membrane-spanning domain (TM) and a cytoplasmic C-terminal domain. The total number of amino acids of each member of the family is indicated. The % of similarity/identity with respect to Cav-1 are also indicated. Based on their functional interaction with heterotrimeric G-proteins, caveolins show either a GDP-dissociation inhibitor activity (GDI) or a GTP-ase activating protein activity (GAP). Figure taken and modified from (Schlegel et al., 1998).

The regions flanking the hydrophobic transmembrane domain, the N-terminal membrane attachment domain (N-MAD) and the C-terminal MAD (C-MAD), allow a high affinity binding to the membranes. During caveolae formation, caveolin undergoes two stages of oligomerisation. Shortly after synthesis, interaction between the oligomerisation domains (OD) allows the formation of 300-350 kDa homo-oligomers in the endoplasmic reticulum, each containing 14-16 caveolin monomers. At a later stage the homo-oligomers interact with each other through the terminal domain (TD) in the C-terminus and form clusters of some 25-50 nm in diameter (Sargiacomo et al., 1995; Scheiffele et al., 1998; Song et al., 1997).
Figure 1.13. Caveolin-1 membrane topology. Two dimers of Cav-1 are drawn bound to the cytoplasmic face of the membrane. The central hydrophobic domain, transmembrane domain (TM) is believed to insert into the membrane. The interaction with the membrane is completed through a tight association between the N-terminal membrane attachment domain (N-MAD) and the C-terminal MAD (C-MAD). Homo-oligomerisation is mediated by the oligomerisation domain (OD) in the N-terminal region of the molecule. Adjacent homo-oligomers interact via the terminal domain (TD) in the C-end (Schlegel et al., 1998).

1.5.3 Caveolin and signal transduction

The discovery that many caveolae resident proteins are signalling molecules led to the suggestion that caveolae are membrane compartments where signal transduction events occur (Lisanti et al., 1994). This idea was confirmed with the observation that Cav-1 interacts with and regulates the activity of different caveolae-associated proteins. Mapping studies in Cav-1 demonstrated that this interaction is mediated by a short sequence overlapping with the homo-oligomerisation domain. This sequence, residues 82-101, has been termed the caveolin scaffolding domain (CSD) (Li et al., 1995; Song et al., 1996). See Figure 1.14.
Figure 1.14. The caveolin scaffolding domain. A schematic representation of the sequences involved in the interaction between caveolin-1 and signalling molecules. The caveolin-binding sequence motifs \( \Phi X \Phi XXXX\Phi, \Phi XXXX\Phi XXX\Phi, \) (where \( \Phi \) is an aromatic residue Trp, Phe, or Tyr and \( X \), any other aminoacid) present in five different caveolin-associated proteins are shown (Schlegel and Lisanti, 2001).

Using the CSD as receptor and random peptides sequences displayed at the surface of bacteriophage, three different motifs that bind to the CSD have been described. These motifs, \( \Phi X \Phi XXXX\Phi, \Phi XXXX\Phi XXX\Phi, \) and \( \Phi X \Phi XXXX\Phi XXX\Phi, \) (where \( \Phi \) is an aromatic residue Trp, Phe, or Tyr and \( X \), any other amino acid) are present within most caveolin-associated proteins (Couet et al., 1997). Many different signalling proteins found in caveolae and expressing the caveolin-binding motif have been reported and some of these include: receptor tyrosine kinases and their downstream targets (EGFR, c-Neu, H-Ras, MEK, ERK); non receptor tyrosine kinases (Src and Fyn); G-protein coupled receptors and their downstream signalling molecules (endothelin receptor, various \( G_\alpha \) subunits, adenylyl cyclase, cyclic AMP-dependent protein kinase) and regulated enzymes (endothelial nitric oxide synthase, eNOS) (Okamoto et al., 1998; Schlegel and Lisanti, 2001). Other evidence of the importance of caveolae as a specialised
membrane domain where signal transduction events take place is the observation that hormones as diverse as insulin, interleukin 2, nerve growth factor, TGF-beta1, and thyroid stimulating hormone all act on their target cells via the common secondary messenger IPG. Since caveolae are highly enriched with GPI linked proteins, a known source for IPG, this suggests that caveolae may some how serve the crucial functions of concentrating the substrate and internalizing the product (Anderson, 1993; Parpal et al., 1995).
THESIS AIMS

Because some controversy exists about the actual number and identity of genes coding for GPI-PLD, the aim of this thesis is to establish whether one or more genes encode for mouse GPI-PLD. In order to investigate the sources of GPI-PLD, particular attention will be focused on the analysis of RNA expression in different tissues and cell lines. To establish if GPI-PLD is associated with pathological conditions, its expression level will be also analysed in tissues derived from different mice strains that constitute animal models of autoimmune diseases.

It has been suggested that GPI-PLD could play an important role in the regulation of the expression of GPI-anchored proteins and the generation of IPG second messenger molecules. It is considered that one important step to study the potential regulatory roles of GPI-PLD is to determine the intracellular site where this enzyme is located and the possible associations with other molecules. In order to study the GPI-PLD activity and intracellular localisation, plasmid vectors coding for GPI-PLD recombinant fusion proteins will be constructed and used for co-transfection experiments. The analysis of cells co-expressing GPI-PLD and caveolin-3 will be undertaken in order to determine whether GPI-PLD is located in the membrane structures termed caveolae, which are rich in GPI-anchored proteins.
CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Laboratory Reagents

Chemicals and organic solvents were purchased from BDH Chemicals (Dorset, UK), Life Technologies (Paisley, UK), or Sigma Chemical Co. (Dorset, UK). Bacteriological agar, yeast extract and tryptone were purchased from Difco Laboratories (Paisley, UK). Double-distilled Milli-Q water (Millipore, Gloucestershire, UK) was used in preparing all solutions. Culture media and solutions for DNA work were sterilised by autoclaving at 103 kPa (15 lb/inch²) for at least 20 min. Thermolabile solutions were filter-sterilised through 0.2 μm Acrodisc syringe filters (Pall, Michigan, USA). All culture media, serum and supplements for growing mammalian cells were purchased from Gibco (Paisley, UK).

All solutions, with exception of Tris-HCl, used in RNA work were prepared using diethylpyrocarbonate (DEPC)-treated water. Solutions were prepared by adding DEPC to a final concentration of 0.1% v/v, leaving the solution overnight at room temperature and removing excess DEPC by autoclaving.

Restriction and modifying enzymes were purchased from Promega (Southampton, UK), New England Biolabs (Hertfordshire, UK) and Stratagene (Cambridge, UK). M-MLV Reverse transcriptase, oligo-dT, ribonuclease inhibitor RNasin, alkaline phosphatase, Taq polymerase and Pfu polymerase were purchased from Promega (Southampton, UK). Antibiotics were purchased from Sigma Chemical Co. (Dorset, UK). Kodak X-OMAT AR and Biomax MR autoradiograph films were obtained from Sigma Chemical Co. (Dorset, UK).
2.1.2 Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>ACTTATTGTCA2AG7TTAGACTTATTTTG</td>
<td>S</td>
<td>ATGACTCTTAAAACCTCCATTTGCCACCCCT</td>
</tr>
<tr>
<td>T7</td>
<td>GTATACGACTGACTAGCGG</td>
<td>SP6</td>
<td>GTAATTTAGTGACACTTAG</td>
</tr>
<tr>
<td>S'BamHI</td>
<td>CGGATCCGGCCCTCCCTGAGTTTCTG</td>
<td>3'Sal I</td>
<td>ACGGCTGAAGCTCTGATGAAACC2TAGG</td>
</tr>
<tr>
<td>Sac 35</td>
<td>AGACTGCAAGAGAGGCGGCTTCTCCAGG</td>
<td>Sal 33</td>
<td>ACGGCTGACAAAAGGCCTCCA2GAAA</td>
</tr>
<tr>
<td>V5'</td>
<td>ACGGCTGATGCTCTCCTCCAAGCATTTA</td>
<td>V3'</td>
<td>ACGGCTGAGCTGCTGATGAAACC2TAGG</td>
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<td>Sac 55</td>
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<td>Sal 53</td>
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<tr>
<td>PLD900s</td>
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<td>PLD900r</td>
<td>ACGGCTGACAAAAGGCCTCCA2GAAA</td>
</tr>
<tr>
<td>PLD500s</td>
<td>TCTGATCTGAGATATCCCTACCTGACC</td>
<td>PLD500r</td>
<td>ACGGCTGACAAAAGGCCTCCA2GAAA</td>
</tr>
<tr>
<td>II5'</td>
<td>ACGGCTGATGCTCTCCTCCAAGCATTTA</td>
<td>II3'</td>
<td>ACGGCTGACAAAAGGCCTCCA2GAAA</td>
</tr>
<tr>
<td>P418</td>
<td>AATTCCAGGGCGGATGACG</td>
<td>P432</td>
<td>GATCCGCTGACTGCTGATGAAACC2TAGG</td>
</tr>
</tbody>
</table>

Table 2.1. Primers used in this work. All the primers were synthesised by Genosys (Cambridgeshire, UK). (s) sense primer; (r) antisense primer.

2.1.3 Bacteria

<table>
<thead>
<tr>
<th>Bacteria Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacf32ZD M15 Tn10 (Tet')].</td>
</tr>
<tr>
<td>XL10-Gold</td>
<td>Tetf32 (mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacf32ZDM15 Tn10 (Tet') Amy Cam'].</td>
</tr>
</tbody>
</table>

Table 2.2. Genotype of *E.coli* bacteria strains used in this work.
2.1.4 Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript SK⁺</td>
<td>Vector designed for high-resolution restriction mapping, sequencing and in vitro transcription. It features a large MCS flanked by T3 and T7 RNA polymerase promoters, ampicillin resistance in E.coli and blue/white colour screening.</td>
<td>Stratagene (Short et al., 1988)</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>Designed for high expression in mammalian cell lines. It features CMV enhancer-promoter, a large multiple cloning site, BGH polyadenylation signal, neomycin resistance, SV40 origin for episomal replication in cells expressing the large T antigen, and ampicillin resistance gene for maintenance in E.coli.</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pcDNA3.1/ Myc-His A</td>
<td>In addition to the properties of pcDNA3, this vector contains a myc epitope (E-Q-K-L-I-S-E-E-D-L) for detection with anti-myc antibodies, and a C-terminal poly histidine tag for purification.</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCRScrypt SK (+)</td>
<td>In addition to the properties of pBluescript SK (+) plasmid, this vector allows cloning of blunt-ended PCR products generated with Phu DNA polymerase.</td>
<td>Stratagene (Liu et al., 1992)</td>
</tr>
<tr>
<td>pEGFP-N3</td>
<td>Designed to synthesise fusion proteins with the GFP in the C-end. Addition of GFP allows localisation of fusion proteins in vivo. It features CMV promoter, HSV-TK polyadenylation signal, SV40 origin of replication and kanamycin resistance.</td>
<td>Stratagene (Chalfie et al., 1994)</td>
</tr>
<tr>
<td>pPROEX HT</td>
<td>Vector designed for the expression of foreign proteins in E.coli. The expressed proteins are fused to a 6-histidine sequence for affinity purification.</td>
<td>Gibco (Polayes, 1996)</td>
</tr>
<tr>
<td>pTri-β-actin Mouse Mouse</td>
<td>This vector contains a 250 bp fragment of the mouse actin (β-actin) gene, in the antisense orientation. The vector has tandem SP6,T7, and T3 promoters to synthesise transcripts.</td>
<td>Ambion (Melton et al., 1984)</td>
</tr>
<tr>
<td>p3XFlag-CMV-8</td>
<td>Designed to express N-terminal 3XFlag fusion proteins in mammalian cells to be detected with anti-Flag antibodies. The vector encodes 3 adjacent Flag epitopes (D-Y-K-X-X-D), where X is any amino acid) upstream of the MCS. It features a CMV promoter, SV40 origin and ampicillin resistance gene.</td>
<td>Sigma (Herman et al., 2000)</td>
</tr>
<tr>
<td>pSPORT 1</td>
<td>Vector for cDNA cloning, in vitro transcription, sequencing and subtraction library procedures. Contains a MCS with sites for 19 restriction endonucleases, flanked by SP6 and T7 RNA polymerase promoters. Ampicillin-resistance gene.</td>
<td>Gibco (Balbas et al., 1986)</td>
</tr>
<tr>
<td>pSV40-PLAP</td>
<td>Vector encoding for the GPI-anchored form of human placental alkaline phosphatase. It features a CMV promoter and ampicillin resistance.</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. Plasmids used and generated in this thesis and their main properties. MCS, multiple cloning site; CMV, cytomegalovirus; BGH, bovine growth hormone; GFP, green fluorescent protein; HSV-TK, herpes simplex virus-thymidine kinase, GHGP, German Human Genome Project.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript SK'/5' AS</td>
<td>Vector containing the 434-bp GPI-PLD fragment (from nucleotide 52 to 486) used to generate an RNA antisense probe for the GPI-PLD 5' end.</td>
<td>This thesis</td>
</tr>
<tr>
<td>SK'/middle part AS probe</td>
<td>Vector containing the 384-bp GPI-PLD fragment (from nucleotide 1009 to 1393) used to generate an RNA antisense probe for the middle part of GPI-PLD.</td>
<td></td>
</tr>
<tr>
<td>pCRIIP1 SK'/3' as probe</td>
<td>Vector containing the 278-bp GPI-PLD fragment (from nucleotide 2713 to 2994) used to generate an RNA antisense probe for the GPI-PLD 3' end.</td>
<td>This thesis</td>
</tr>
<tr>
<td>pSPORT1-GPI-PLD</td>
<td>Vector containing the entire GPI-PLD sequence obtained from a mouse liver library.</td>
<td>GHGP</td>
</tr>
<tr>
<td>pPROEX HtGFP::GPI-PLD</td>
<td>Vector containing the entire GPI-PLD coding sequence without the stop codon.</td>
<td>This thesis</td>
</tr>
<tr>
<td>pSPORT1-GPI-PLD</td>
<td>Vector containing the GPI-PLD sequence lacking the signal sequence.</td>
<td>This thesis</td>
</tr>
<tr>
<td>pGPS</td>
<td>Vector used as transposon donor for the generation of GPI-PLD templates for sequencing. The vector confers chloramphenicol resistance and contains specific primer-binding sites for sequencing.</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pHR CMV-IR-EGFP</td>
<td>This viral vector contains an internal ribosome entry sequence (IRES) and the sequence coding for green fluorescent protein. Used to generate a GPI-PLD antisense-coding plasmid.</td>
<td>Clontech (Zufferey et al., 1997)</td>
</tr>
<tr>
<td>pEGFP-N3</td>
<td>Plasmid coding for GFP. Allows generation of fusion proteins with GFP attached to the carboxyl end.</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCDNA3/GPI-PLD</td>
<td>Plasmid expressing recombinant GPI-PLD. See Chapter 4.</td>
<td>This thesis</td>
</tr>
<tr>
<td>pCDNA3/GPI-PLD/Myc-His A</td>
<td>Plasmid expressing a fusion protein with the Myc tag attached to the carboxyl end of GPI-PLD. See Chapter 4.</td>
<td>This thesis</td>
</tr>
<tr>
<td>pGPI-PLD/EGFP-N3</td>
<td>Plasmid expressing a fusion protein with GFP attached to the carboxyl end of GPI-PLD. See Chapter 4.</td>
<td>This thesis</td>
</tr>
<tr>
<td>p3XFlag-CMV8-GPI-PLD</td>
<td>Plasmid expressing a fusion protein with the 3XFlag tag attached to the amino end of GPI-PLD. See Chapter 4.</td>
<td>This thesis</td>
</tr>
</tbody>
</table>

Table 2.3. Cont. Plasmids used and generated in this thesis and their main properties.
### 2.1.5 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Concentration</th>
<th>Isotype</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Flag monoclonal (M2) antibody</td>
<td>Sigma</td>
<td>4 mg/ml</td>
<td>IgG1</td>
<td>ICS 1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>Clontech</td>
<td>1 mg/ml</td>
<td>IgG1</td>
<td>WB 1:1000</td>
</tr>
<tr>
<td>Anti-HA (HA-7 clone) monoclonal antibody</td>
<td>Sigma</td>
<td>-</td>
<td>IgG1</td>
<td>ICS 1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>Purified mouse immunoglobulins</td>
<td>Southern Biotechnol</td>
<td>0.1 mg/ml</td>
<td>-</td>
<td>ICS 1:100</td>
</tr>
<tr>
<td>Rabbit anti-mouse Igs HRP- conjugated</td>
<td>Dako</td>
<td>1.3 mg/ml</td>
<td>IgGs</td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>Rabbit anti-mouse Igs RPE- conjugated</td>
<td>Dako</td>
<td>0.5 mg/ml</td>
<td>(Fab')₂</td>
<td>ICS 1:250</td>
</tr>
<tr>
<td>Rabbit anti-mouse Igs FITC- conjugated</td>
<td>Dako</td>
<td>0.5 mg/ml</td>
<td>(Fab')₂</td>
<td>ICS 1:250</td>
</tr>
<tr>
<td>Hamster monoclonal anti-mouse CD14 (G5A10)(^A)</td>
<td>Dr. Landmann</td>
<td>6.89 mg/ml</td>
<td>-</td>
<td>ELISA 1:300</td>
</tr>
<tr>
<td>Rabbit anti-mouse CD14 biotinylated</td>
<td>Dr. Landmann</td>
<td>1 mg/ml</td>
<td>-</td>
<td>ELISA 1:5000</td>
</tr>
<tr>
<td>Rat anti-mouse CD14</td>
<td>PharMingen</td>
<td>0.5 mg/ml</td>
<td>IgG1, κ</td>
<td>WB 1:1000</td>
</tr>
<tr>
<td>Hamster anti-mouse TNF(α)</td>
<td>Dr. De Souza</td>
<td>-</td>
<td>-</td>
<td>ELISA 1:1000</td>
</tr>
<tr>
<td>Rabbit anti-mouse TNF(α)</td>
<td>Genzyme</td>
<td>-</td>
<td>-</td>
<td>ELISA 1:300</td>
</tr>
<tr>
<td>Goat anti-rabbit HRP-conjugated</td>
<td>Dako</td>
<td>0.3 mg/ml</td>
<td>-</td>
<td>ELISA 1:2000</td>
</tr>
</tbody>
</table>

Table 2.4. A list of antibodies used in this work. Indicated here, are the sources of antibodies, the stock concentration, isotype and working dilutions used in intracellular staining (ICS), Western blot (WB), and enzyme-linked immunosorbent assay (ELISA). \(^A\), antibodies obtained from Dr. Landmann, Division of Infection Diseases, University Hospital, Basel. B, antibody obtained from Dr. De Souza, Department of Immunology and Molecular Pathology, UCL.
2.1.6 Cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Organism Tissue</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21 (ATCC CCL-10)</td>
<td>Syrian golden hamster/kidney</td>
<td>Caveolin-3 negative cell line.</td>
</tr>
<tr>
<td>COS-7 (ATCC CRL-1651)</td>
<td>African green monkey/kidney</td>
<td>Positive for the SV40 large T antigen.</td>
</tr>
<tr>
<td>HepG2 (ATCC HB-8065)</td>
<td>Human/liver</td>
<td>Hepatocellular carcinoma cell line. Expresses Apo A-I.</td>
</tr>
<tr>
<td>NIT-1 (ATCC CRL-2055)</td>
<td>NOD/Lt mouse/pancreas (beta cell)</td>
<td>Derived from an insulinoma. Positive for the SV40 large T antigen.</td>
</tr>
<tr>
<td>293T (Gene Hunter Q401)</td>
<td>Human/kidney</td>
<td>Positive for the SV40 large T antigen.</td>
</tr>
<tr>
<td>RAW264.7 (ATCC TIB-71)</td>
<td>Mouse/monocyte-macrophage</td>
<td>GPI-PLD and CD14-expressing cell line.</td>
</tr>
<tr>
<td>P388 (ATCC TIB-71)</td>
<td>Mouse/monocyte-macrophage</td>
<td>GPI-PLD and CD14-expressing cell line.</td>
</tr>
</tbody>
</table>

Table 2.5. Cell lines used in this study. The main properties and organisms where the cell lines where derived from are indicated. Cells were analysed every month for mycoplasma infection using a PCR-based mycoplasma detection kit (ATCC). All cells tested negative.

2.1.7 Solutions

**Antibiotic stock solutions:** Ampicillin (100 mg/ml) and kanamycin (30 mg/ml), prepared in double distilled water and filter-sterilised. Tetracycline (10 mg/ml) and chloramphenicol (20 mg/ml) prepared in 80% ethanol, no filter-sterilising required.

**Buffer A (genomic DNA):** 100 mM EDTA, 100 mM NaCl, 25 mM Tris-HCl pH 8.0.

**Buffer D:** 6M guanidinium isothiocyanate, 0.75M sodium citrate, 10% sарcosyl, 1.4 M 2-β-mercaptoethanol.
CHAPTER 2, MATERIALS AND METHODS.

Buffer I (competent cells): 100 mM RbCl, 50 mM MnCl, 30 mM CH₃COOK, 10 mM CaCl₂, 15% (v/v) glycerol. Adjusted to pH 5.8 with 0.2 N acetic acid. Filter-sterilised and kept at 4°C.

Buffer II (Competent cells): 10 mM MOPS (pH 7.0 adjusted with NaOH), 10 mM RbCl, 75 mM CaCl₂, 15% (v/v) glycerol. Filter-sterilised.

Carbonates buffer: 35 mM NaHCO₃, 15 mM Na₂CO₃. Adjusted to pH 9.6.

Church's buffer: 7% SDS, 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 0.1μg/ml yeast RNA, 1% BSA.

Chloroform/isoamyl alcohol (24:1): Mix 24 parts (volumes) of chloroform and 1 part of isoamyl alcohol, and protected from light.

Cocktail of protease inhibitors: 300 μM aprotinin, 0.5 mM EDTA, 1 μM pepstatin, 500 μM PMSF, and 50 μM leupeptin.

DEAE-dextran/chloroquine solution: PBS containing 10 mg/ml DEAE-dextran and 2.5 mM chloroquine.

DNA loading buffer: 0.25% bromophenol blue, 50 mM EDTA, 10 mM Tris-HCl (pH 7.5), 10% Ficoll 400, 0.25% xylene cyanol.

GPS buffer (10X): 250mM Tris-HCl (pH 8.0), 20 mM DTT, 20 mM ATP, bovine serum albumin (BSA) 500 mg/ml.

GTE solution: 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA.

HBS buffer (10X): 8.18% NaCl, 5.94% HEPES and 0.2% Na₂HPO₄. Stored at 4°C as 10X concentrated stock. This buffer was diluted to 2X concentration, the pH was adjusted to exactly 7.12 and filter-sterilised through 0.2 μm filters for working stock.

Hoegness freezing medium (10X): 36 mM K₂HPO₄, 13 mM KH₂PO₄, 20 mM sodium citrate, 10mM MgSO₄•7H₂O, 44% glycerol. Filter sterilised and kept at 4°C.

HRP substrate buffer: Two tablets of o-phenylenediamine dihydrochloride were dissolved in 10 ml of carbonates buffer containing 5 mM MgCl₂.

Ligase buffer (10X): 300 mM Tris-HCl pH 7.8, 100 mM MgCl₂, 100 mM DTT, and 10 mM ATP.

Luria-Bertani (LB) media: 10 g tryptone, 5 g yeast extract and 5 g NaCl in 1L of deionised water. Adjusted to pH 7.5 with 10 N NaOH and autoclaved.

MBS buffer: 25 mM MES (pH 6.5), 0.15 M NaCl.

Lysis Mix: 200 mM NaOH, 1% SDS.
M-MLV reaction buffer (5X): 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂, and 50 mM DTT

Mowiol mounting solution: 2.4 gram Mowiol 4.88 (Calbiochem, California, USA) were added to 6 ml of glycerol and stirred to mix. 6 ml H₂O were added and left 2 h at room temperature. 12 ml 0.2 M Tris, pH 8.5 were added and the solution incubated in hot water (50°C) for up to 1 h and then centrifuged at 5000 g for 15 min. The supernatant was collected and 1 granule of p-phenylenediamine (antiquench) to 1 ml Mowiol was added. The solution was stored in small aliquots in glass tubes, at -70°C. Once thawed, aliquots were kept at -20°C for 1 week.

PBS buffer (1X): 1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 154 mM NaCl. Adjust pH to 7.2-7.4 and autoclave.

PBS-Tween 20: PBS buffer containing 0.05% Tween 20.

PCR polymerase reaction buffer (1x): 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 1% Triton X-100, 50% glycerol.

Phenol: Phenol was equilibrated to pH>7.8 by shaking with an equal volume of 0.5 M Tris-HCl (pH 8.0). The solution was left to allow the separation of two phases. The top aqueous phase was removed and the process was repeated twice with 0.5 M Tris-HCl (pH 8.0) and once with 0.1 M Tris-HCl (pH 8.0). After the last equilibration, a thin layer of Tris-HCl buffer was left on top of equilibrated phenol and 0.1% of hydroxyquinoline was added. The solution was kept in dark at 4°C.

Phenol-chloroform (1:1): Equal volumes of 24:1 chloroform/isoamyl alcohol and equilibrated phenol were mixed. 0.1% of hydroxyquinoline was added and the solution kept in dark at 4°C.

PLAP assay solution: 1.33 M diethanolamine (pH 9.8), 0.66 mM MgCl₂, 13.33 mM p-nitrophenylphosphate and 10 mM homoarginine.

Polyacrylamide resolving gel (10%): 9.73% acrylamide monomer, 0.26% bis-acrylamide, 375 mM Tris (pH 8.0), 0.1% SDS, 0.05% (NH₄)₂S₂O₈ (ammonium persulfate, APS), and 0.05% TEMED.

Polyacrylamide stacking gel (5%): 4.96% acrylamide monomer, 0.13% bis-acrylamide, 375 mM Tris (pH 8.0), 0.1% SDS, 0.05% (NH₄)₂S₂O₈ (APS), and 0.05% TEMED.

PSG buffer: 53 mM Na₂HPO₄, 3 mM NaH₂PO₄, 50 mM NaCl, 55 mM L-glucose. Adjusted to pH 8.0.

RNA electrophoresis buffer (20X): 0.8 M MOPS (pH 7.0), 0.2 M CH₃COONa 3H₂O, 0.02 M EDTA. Adjusted pH to 7.2.

RNA loading dye (2X): 0.5X MOPS buffer, 25% formaldehyde (from 37% solution), 65% de-ionised formamide, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.025% ethidium bromide, 0.5mM EDTA, 10% glycerol.
CHAPTER 2. MATERIALS AND METHODS.

RPA digestion buffer: 300 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA.

RPA hybridisation buffer: 40 mM PIPES (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, 80% formamide.

RPA loading buffer: 80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue.

SDS loading buffer (2X): 100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol. 200 mM dithiothreitol was added before use.

SDS-PAGE running buffer: 0.025 mM Tris (pH 6.8), 192 mM glycine, 0.1% SDS. Adjusted to pH 8.0.

Sequencing 6% acrylamide gel (100 ml): 5.7% acrylamide, 0.3% bis-acrylamide, 42% urea. De-ionised water was added up to 100ml, 500 µl 10% APS and 50 µl TEMED.

Sequencing stop solution (2X): 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF.

Silica solution: A solution (100 mg/ml) was prepared in PBS and allowed to settle for 2 h. The supernatant was removed and the procedure repeated. After centrifugation (2000 g for 2 min) the silica was resuspended in 3 M NaI at 100 mg/ml. This solution was used as DNA-binding matrix.

TAE buffer: 40 mM Tris-acetate, 1 mM EDTA. Adjusted to pH 7.2 and autoclaved.

TBE buffer: 90 mM Tris, 2 mM EDTA, 90 mM boric acid, adjusted to pH 8.0.

TBS: 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, adjusted to pH 7.6.

TBS-Tween 20: TBS buffer containing 0.1% Tween 20.

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

TEN buffer (1X): 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl.

Transcription buffer 5X: 200 mM Tris-HCl (pH 7.5), 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl.

Versene: 0.1 M NaCl, 1.5 mM KH₂PO₄, 2.7 mM KCl, 8 mM Na₂HPO₄, 1 mM EDTA, and 5.6 mM glucose.

VSG buffer A: 150 mM NaCl, 10 mM HEPES (pH 7.0), and 0.1% NaN₃.

VSG Labelling medium: RPMI 1640 culture medium supplemented with 1 mg/ml of fatty acid-free BSA and 25 mM HEPES (pH 7.4).

VSG Lysis solution: 10 mM sodium phosphate containing 1 µg/ml leupeptin hydrochloride (from a 5 mg/ml stock solution), 0.1 mM TLCK, (prepared as a 100 mM...
solution on the day of use), and 5 mM sodium p-chloromercuriphenylsulfonic acid, (from a 100 mM stock solution in 0.1 M NaOH).

**VSG substrate mixture:** 40 mM Tris/maleate (pH 7.0), 0.1 % NP-40 and 5 000 cpm $[^3]$H]VSG.

**Washing buffer (Purification of DNA fragments):** 50 mM NaCl, 10 mM Tris-HCl, 2.5 mM EDTA, 50% v/v ethanol.

**Western Blotting transfer buffer:** 39 mM glycine, 48 mM Tris-base, 0.037% SDS, 20% methanol. Adjusted to pH 8.0.
2.2 DNA METHODOLOGY

2.2.1 Bacterial cultures

Liquid cultures of ampicillin, kanamycin and/or tetracycline resistant bacteria were incubated at 37°C over night in Luria-Bertani (LB) media containing ampicillin (100 μg/ml), kanamycin (30 μg/ml) and/or tetracycline (10 μg/ml) in an orbital shaker incubator at 200 rpm. Starter cultures were prepared by inoculating 5 ml of LB media, and appropriate antibiotic with a single colony for 5-8 h. Larger volume cultures for plasmid preparations were inoculated with 1:1000 (v/v) of a starter culture.

Plate cultures of antibiotic-resistant bacteria incubated at 37°C on LB medium supplemented with 2% of bacto-agar, containing ampicillin (100 μg/ml), kanamycin (30 μg/ml) and/or tetracycline (10 μg/ml). Blue/white selection was performed on plates coated with 100 μl of 100 mM Isopropyl β-D-thiogalactopyranoside (IPTG) and 50 μl of 50 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Insight Biotechnology, Wembley, UK).

Long term stocks were prepared from an overnight liquid culture of a single bacteria derived from a fresh selective plate. 900 μl of bacteria suspension was mixed with 100 μl of 10X Hoegness freezing medium. Samples were dispensed in cryogenic vials (Nalgene, Rochester NY, USA) and stored at -80°C.

2.2.2 Preparation of competent bacteria

A single colony of E.coli from a fresh plate was incubated overnight in 5 ml of in LB medium. The following day, the culture was diluted 20 times in 100 ml of LB medium at 37°C and grown until an OD₅₅₀ of 0.48 was reached. At this point, the cells were chilled on
ice for 10 min and then centrifuged at 5000 g for 15 min at 4°C. The pellet was resuspended in 30 ml of ice-cold Buffer I. The cells were centrifuged for 10 min at 5000 g and 4°C and then gently resuspended in 4 ml of Buffer II and left in ice for 15 min. Finally, the cells were dispensed in 200-μl aliquots and stored at -80°C.

2.2.3 Transformation of competent bacteria

Competent *E. coli* were thawed on ice, mixed with the plasmid DNA (0.5 μg) and incubated on ice for 30 min. The cells were heat-shocked for 90 seconds at 42°C and returned to ice for 2 min. 450 μl of LB medium were added, and the cells were incubated at 37°C with vigorous agitation for 1 h. The cells were centrifuged at 4000 g for 30 seconds and 300 μl of the supernatant was discarded. The cells were resuspended and spread onto selective LB agarose plates and incubated overnight at 37°C.

2.2.4 Preparation of plasmid DNA

a) Small-scale preparation (mini-prep)

1.5 ml from a 5 ml overnight culture was centrifuged for 1 min at 5 000 g. The pellet was resuspended in 100 μl of GTE solution and incubated at room temperature for 5 min. 200 μl of lysis mix was added, and the tube was carefully inverted several times to mix the solutions and incubated on ice for 5 min. Finally, 150 μl of 3M sodium acetate (pH 5.6) was added, the tube inverted a couple of times and incubated on ice for 5 min. The precipitate was removed by centrifuging for 10 min at 10 000 g at 4°C. The supernatant was transferred to a new tube, extracted twice with an equal volume of a phenol/chloroform (1:1 v/v) solution and once with an equal volume of chloroform. The aqueous layer containing the DNA was removed and precipitated with 2.5 volumes of ethanol. The DNA
was collected by centrifugation at 10 000 g and 4°C for 10 min. The pellet was washed with 70% ethanol and dissolved in 50 μl of TE buffer. An aliquot of 5 μl was digested with restriction enzymes.

Alternatively, very high-quality plasmid DNA for sequencing was extracted and purified from 5-ml liquid overnight cultures using the QiagenSpin Miniprep kits (Qiagen, Sussex, UK), according to the standard protocol of the manufacturers.

b) Large-scale preparation (midi-prep)
Plasmid DNA was obtained from large-volume overnight cultures following the alkaline lysis protocol (Sambrook et al., 1989). Bacteria from a 250-ml overnight culture were harvested by centrifugation at 5 000 rpm for 15 min at 4°C in a Beckman centrifuge (model J-6B). The pellet was resuspended in 6 ml of ice-cold GTE solution and incubated on ice for 10 min. Bacteria were lysed by adding 12 ml of lysis mix, carefully mixed and incubated for 20 min on ice. The solution was neutralised by adding 7.5 ml of 3M sodium acetate (pH 5.6), mixed by gentle inversion and incubated 20 min on ice. The cellular debris was removed by centrifuging for 15 min at 10 000 g. The supernatant was transferred to a new tube and precipitated with 0.6 volumes of isopropanol. After centrifuging at 10 000 g for 20 min at 4°C, the pellet was resuspended in 2.5 ml of TE buffer. Three volumes of ice-cold 4M LiCl was added and incubated for 30-60 min on ice. Following centrifugation for 20 min at 10 000 g, DNA was precipitated with 0.6 volumes of isopropanol. Precipitated DNA was resuspended in 800 μl of TE and then treated with 20 μg/ml of DNAase-free RNAase for 1 h at 48°C. The solution was extracted twice with an equal volume of a phenol/chloroform solution and once with one volume of chloroform.
The DNA in the aqueous phase was precipitated by adding 80 µl of 4M NaCl and 500 µl of 13% PEG, and incubating on ice for 1 h. The DNA was recovered by centrifugation at 10 000 g and 4°C for 15 min, washed with 70% ethanol, dissolved in TE and extracted once with a similar volume of chloroform. After adding 0.25 volumes of 7.5 M ammonium acetate, the DNA in the aqueous phase was precipitated with two volumes of absolute ethanol, washed with 70% ethanol and resuspended in TE buffer. 5 µl of DNA solution was diluted 1:200 in H2O to determine the concentration by measuring the optical density in a spectrophotometer. An absorbance of 1.0 at 260 nm is equivalent to a concentration of approximately 50 µg/ml of double-stranded DNA (Sambrook et al., 1989). DNA was stored at -20°C.

2.2.5 Digestion of DNA with restriction enzymes

The digestion of plasmid DNA was carried out according to the manufacturer's instructions. 1 µg (for plasmid analysis) or 20 µg (for purification of plasmid fragments) of plasmid DNA were digested in a final volume of 20 or 200 µl. The volume of the enzyme(s) used never exceeded 10% of the total volume and the final concentration of the digestion buffer was 1X. Digestions were carried out at 37°C in a water bath for 2 h, unless stated otherwise by manufacturer.

For Southern blot analysis 10-20 µg of genomic DNA was digested for 6-8 h at 37°C in a final volume of 50 µl. The complete digestion of genomic DNA was analysed in a parallel digestion on an aliquot of 5 µl of the digestion mix in the presence of 1 µg of unmethylated lambda (λ) phage (Promega, Southampton, UK).

The digestion products were analysed by electrophoresis in horizontal agarose gels. DNA samples were mixed 1:4 ratio with DNA loading buffer and loaded in 1-2% agarose
CHAPTER 2. MATERIALS AND METHODS.

(Bio/Gene Limited, Cambridgeshire, UK) gels. Electrophoresis-grade agarose was dissolved in 1X TAE buffer and ethidium bromide (0.5 μg/ml final concentration) was added. Plasmid DNA was separated under a constant voltage of 75 V for 45 min on 1X TAE buffer. After running, DNA samples were identified under an UV-light trans-illuminator (302 nm wavelength) and sized by comparing its mobility to fragments of known size (1kb ladder or 100 bp ladder, Fermentas, Tyne and Wear, UK).

2.2.6 Purification of DNA fragments by electrophoresis in agarose gels

After DNA was separated in agarose TAE gels, the desired fragments were identified in the UV-light transiluminator and excised from the gel. The agarose fragments (0.2-0.4 g) were solubilised in 3 volumes of a 6 M NaI solution at 45-55°C. After the agarose was completely dissolved, 10-20 μl of a 100 mg/ml silica solution was added. The solution was incubated at 4°C for 30 min with constant agitation. The silica matrix with the bound DNA was centrifuged and washed three times with 500 μl of Washing buffer, dried and resuspended in 20-50 μl of TE buffer. The purified fragment was checked by agarose gel electrophoresis (Boyle and Lew, 1995).

2.2.7 Ligation reactions

After completed digestion of the vector, the restriction enzymes were heat-inactivated for 15 min. 5' ends of the vector were de-phosphorylated by incubation for 30 min at 37°C with calf intestine alkaline phosphatase (CIAP, 0.01 U/pmol of ends) in 1X CIAP reaction buffer in a final volume of 100 μl. The CIAP was heat-inactivated at 75°C for 15 min followed by phenol/chloroform extraction and ethanol precipitation of the DNA. The DNA (0.1 μg) was finally resuspended in TE buffer.
A typical ligation reaction of DNA fragment molecules consisted of 50-100 ng of vector DNA, 20-100 ng of insert DNA, 1 U of T4 DNA ligase and 1X ligase buffer in a final volume of 10-20 μl. The reaction was incubated overnight at 16°C, and 5 μl of the ligation reaction were used for transformation of competent *E. coli*. Alternatively, the Stratagene pCRScript system (Cambridge, UK) was used to ligate PCR-generated DNA fragments into the PCRScript vector.

### 2.2.8 Reverse transcriptase reaction

cDNA was synthesised using the M-MLV reverse transcriptase and following the standard protocol from manufacturer. Briefly, 2 μg of total RNA was resuspended in 13 μl of DEPC-treated water and mixed in a test tube with 2 μl of oligo-dT (0.5 mg/ml). The tube was incubated at 70°C for 5 min and then incubated on ice for 10 min.

The following components were added to the annealed primer/template in the following order: 5 μl of M-MLV 5X reaction buffer (Promega, Southampton, UK), 1.25 μl of each 10 mM dNTP (0.5 mM, final concentration), 1.6 U of ribonuclease inhibitor RNasin, 200 U M-MLV reverse transcriptase, and DEPC-treated water up to a final volume of 25 μl. The reverse transcription reaction was incubated at 42°C for 1h, followed by inactivation of the reverse transcriptase at 95°C for 10 min. The reaction was diluted to 200 μl with DEPC-treated water and 10 μl were used for the polymerase chain reaction.
2.2.9 Polymerase chain reaction (PCR)

50 ng of plasmid template or 10 μl of the diluted reverse transcription reaction were mixed with 50 pmol of sense and antisense primers (0.5 μM final concentration), 1x polymerase reaction buffer (Promega, Southampton, UK), 1.5 mM of MgCl2, 2 μM of each dNTP, and 5 units (U) of Pfu-polymerase. The final volume of the reaction was 100 μl and the amplification program consisted of:

- One segment of DNA denaturation for 4 min at 94 °C.
- A second segment of 30 cycles consisted of denaturation at 94°C for 30 sec followed by an annealing step at an appropriate temperature (usually 5°C below the \( T_m \) \( T_m = 4 \times G/C + 2 \times A/T \)) for 2.5 min, and a final elongation step for 1 to 2.5 min, depending on the PCR product, at 72°C.
- A final segment of elongation for 7 min at 72°C.

The PCR products were analysed and purified by gel electrophoresis.

2.2.10 Generation of templates for sequencing

GPI-PLD clone 19 DNA was sequenced using a Genome Priming System (GPS) (New England Biolabs, Hertfordshire, UK). 2 μl of the 10X GPS buffer was mixed with 0.02 μg of the pGPS plasmid (transposon-donor), 0.08 μg of the GPI-PLD clone 19 and water to a final volume of 18 μl. After mixing, 1 μl of TnsABC* transposase was added, mixed and the solution was incubated 10 min at 37°C to generate the assembly reaction. Magnesium acetate (start solution) was added (15 mM final concentration) to the assembly reaction and further incubated 1 h at 37°C to complete the strand transfer reaction (Figure 2.1).

After heating at 75°C for 10 min, 1 or 10 μl of this reaction was used to transform competent E.coli XL1-Blue. Transformed bacteria were selected using ampicillin and...
chloramphenicol-resistance conferred by the transposon. Analysis with restriction enzymes
SalI and NotI allowed to selected plasmids where the transposon had become inserted
into GPI-PLD, which were used for DNA sequencing. Samples were sequenced using an
ABI PRISM genetic analyser (Applied Biosystems, California, USA).
Population of sequencing templates with randomly inserted transposon.

**Figure 2.1.** Overview of Genome priming system (GPS). The target DNA, mouse GPI-PLD clone 19 inserted into the Sal I/Not I sites of pSPORT1, is incubated in the presence of transposase Tns ABC and a transprimer-donor plasmid, which contains the specific primer-binding sites (in red). A population of DNA sequencing templates with randomly inserted primer-binding sites is generated. After selection with antibiotics, suitable templates for subsequent sequencing are selected by restriction analysis using Not I and Sal I enzymes. The red arrows indicate the direction of sequencing (adapted from the GPS kit instruction manual).
2.2.11 Sequencing

Plasmid DNA for sequencing was purified using a QiagenSpin Miniprep kit (Qiagen, Sussex, UK) and the sequencing reactions were performed using thermo sequenase pre-mixed sequencing systems (Amersham, Buckinghamshire, UK) following the manufacturer instructions. DNA sequencing relies on the PCR synthesis of a new strand of DNA starting at a specific priming site and ending with the incorporation of a chain termination nucleotide. In addition, this sequencing method uses repeated cycles of thermal denaturation, annealing and extension/termination to increase signal level. The fragments obtained during the PCR reactions were separated by electrophoresis on a denaturing acrylamide gel in TBE buffer (Sanger et al., 1977).

The labelling reaction was performed by adding the following to the template solution: 5 mM DTT, 0.5 μM of each dGTP, dCTP and dTTP; 0.8 µl [α-35S] dATP (1000 Ci/mmol), and 10 U of T7 sequenase (DNA polymerase). The solution was mixed and left at room temperature for 3 min. Previously, 2.5 µl of each termination mix ('A', 'C', 'G' and 'T', final concentration of 30 μM for each dATP, dCTP, dGTP and dTTP) were added to eppendorf tubes. When the labelling reaction was complete, 3.5 µl of this solution was transferred to each termination tube, mixed and incubated 5 min at 37°C. The reaction was stopped by adding 4 µl of 2X sequencing stop solution.

The samples were separated by electrophoresis in a vertical 6% acrylamide/8 M urea gel in 1X TBE buffer at 70 watts for 4 h (short run) or 8 h (long run). When electrophoresis complete, the gel was dried at 80°C in a vacuum dryer and exposed overnight at room temperature to an autoradiograph X-OMAT film. Initial sequencing close to the 5' and 3' ends of the GPI-PLD clones isolated from a liver cDNA library was done by using this sequencing protocol.
2.2.12 Liver cDNA library

The cDNA GPI-PLD clones (19, 12 and 9) analysed in this work were obtained after screening a cDNA library with a rat GPI-PLD-specific PCR probe (from Julian Schofield, Molecular Pathology Department, UCL). The library was purchased from the German Human Genome Project and was derived from the liver of a C57BL/10 male mouse. This library contained $5.5 \times 10^5$ clones with inserts within 650 to 4200 bp cloned into the Sal I–Not I sites of the pSPORT 1 vector (Gibco BRL, Paisley, UK).

2.2.13 Genomic DNA preparation

Genomic DNA was extracted from the liver of CBA mice. The tissues kept at -80°C, were thawed in 25 ml of Buffer A and homogenised in a sterile glass homogeniser (Jencons, Bedfordshire, UK). 20% SDS solution containing 10 mM Tris-HCl (pH 8.0) was added to the single cell suspension to a final concentration of 1% SDS. This solution was incubated 10-15 min at 56°C before being extracted with an equal volume of a phenol/chloroform/isoamyl alcohol (25:24:1). The solution was gently mixed until a homogeneous emulsion was formed. Following centrifugation for 15 min at 4°C and 3000 rpm, the aqueous phase was carefully transferred into a new tube and re-extracted with an equal volume of phenol/chloroform/isoamyl alcohol.

DNA was precipitated with 2.5 volumes of isopropanol, spooled out, washed with 70% ethanol and dissolved in 10 ml of TE buffer. The liver DNA was treated with RNAase (20μg/ml final concentration) for 30 min at 37°C. After the RNAase treatment, SDS (final concentration of 0.1%) and proteinase K (100 μg/ml) were added, and the solution was further incubated for 1 h at 56°C.
The solution was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and once with an equal volume of chloroform. Following addition of ammonium acetate (final concentration of 2M), the DNA was precipitated with 2.5 volumes of absolute ethanol. The precipitated DNA was spooled out, washed in 70% ethanol and finally dissolved in 2 ml of TE. The optical density at 260 nm was determined to calculate the concentration.

2.2.14 Southern Blot

10 µg of liver genomic DNA were digested with restriction enzymes and separated overnight at 40 Volts in a 0.7% ethidium bromide-containing agarose gel in 1X TAE buffer. The separated DNA was visualised in an UV-light trans-illuminator before the gel was denatured in 0.4 M NaOH with gently shaking for 40 min at room temperature. The DNA was transferred overnight to a Hybond-N* nylon membrane (Amersham, Buckinghamshire, UK) by capillary blotting with 0.4 M NaOH solution. After transfer, the nylon membrane was washed for 10-15 min with 2X SSC solution before being air-dried and kept at room temperature until hybridisation.

The nylon membranes were pre-hybridised in Church’s buffer (containing 20 µg/ml of sonicated herring DNA) at 65°C for 1-2 h. Membranes were hybridised overnight at 65°C in the same Church’s buffer in the presence of a radioactive DNA probe. The next day, membranes were washed with subsequent changes of 2X SSC, 0.1% SDS; 1X SSC, 0.1% SDS and 0.1X SSC, 0.1% SDS solutions at 65°C until no radioactivity was detectable in the washing solution. After washing, membranes were wrapped in plastic film and exposed for 2-4 days to X-OMAT films at -80°C.
2.2.15 Labelling of radioactive DNA probes

DNA probes were generated using a Rediprime™ II kit (Amersham, Buckinghamshire, UK). 25-50 ng of DNA was resuspended in a final volume of 45 μl of TE buffer. The DNA was denatured by heating to 95-100°C for 5 min, snap cooled on ice for 5 min, and centrifuged. The dissolved DNA was added to the reaction tube, containing a buffered solution of dATP, dGTP, dTTP, exonuclease-free Klenow enzyme and random primers in a dried, stabilised form. 50 μCi of [α-32P] dCTP (370 MBq/ml) were added and the components were mixed. The reaction was incubated at 37°C for 1h, stopped by adding 5 μl of 0.2 M EDTA, and the DNA was purified in a ChromaSpin TE-30 column (Clontech, California USA). Finally, the probe was diluted to 250 μl with TEN buffer, boiled for 5 min at 95°C, snap cooled in ice for 5 min, and added to the hybridisation solution.
2.3 RNA METHODOLOGY

2.3.1 Total RNA extraction

Dissected tissues were snap-frozen in liquid nitrogen. Total RNA was extracted using the guanidinium thiocyanate-phenol-chloroform extraction (MacDonald et al., 1987). The frozen tissues were sonicated in Buffer D. After adding 1/10 volume of 2 M sodium acetate (pH 4.1), the solution was extracted with 1 volume of phenol-chloroform solution (1:1 v/v). The solution was left in ice for 15 min and then centrifuged at 6 000 g for 20 min at 4°C. After centrifugation, the aqueous layer was transferred to a new tube and RNA was precipitated by adding 1 volume of isopropanol for 1 h at -20°C.

The RNA was precipitated by centrifuging 15 min at 10 000 g and 4°C in a Beckman J-6B centrifuge, washed in 70% ethanol and left to dry. The RNA was dissolved in 3 ml of Buffer D and re-extracted with an equal volume of phenol/chloroform solution. The aqueous phase was transferred to a new test tube, an equal volume of isopropanol was added, and the RNA was precipitated for 1 h at -20°C. After centrifugation at 10 000 g for 15 min at 4°C, the supernatant was removed, the pellet washed in 70% ethanol and dissolved in 200 μl of DEPC-treated water. The RNA was re-precipitated for 1 h at -20°C by adding 40 μl of 3M sodium acetate (pH 5.6) and 600 μl of ethanol. The RNA was pelleted by centrifugation at 10 000 rpm for 15 min at 4°C, washed in 70% ethanol and was finally re-suspended in 200 μl of DEPC-treated water. 10μl of this solution was used to determine the concentration and to the remaining solution, 40 μl of 3M sodium acetate (pH 5.6) and 600 μl of ethanol were added. The RNA was stored as ethanol-precipitate at -20°C. RNA concentration was determined by measuring the optical density of a 1:220 dilution in a spectrophotometer at 260 nm. An absorbance of 1.0 at this wavelength equivalent to a concentration of approximately 40 μg/ml of RNA (Sambrook et al., 1989).
2.3.2 Northern Blot Assays

Total RNA extracted from different mouse tissues was separated in a 1% agarose-formaldehyde gel. This gel was prepared by dissolving 1.8 g of agarose in 142 ml of DEPC-treated water in a microwave oven. When agarose was dissolved, the solution was transferred to a water bath at ~65°C and 9 ml of RNA electrophoresis buffer (20X) pH 7.2 was added. After this, 29 ml of formaldehyde (37% solution) was added and the gel was poured into a gel tray in a fume hood and left to set.

20-40 µg of the ethanol precipitated RNA samples were centrifuged at 14 000 rpm for 15 min at 4°C, washed with 70% ethanol and re-suspended in 50 µl of DEPC-treated water. The RNA was mixed with an equal volume of RNA sample buffer and heated at 80°C for 3 min. The samples were placed on ice for 2 min, spun briefly and loaded on the gel.

The gel was run overnight at 40 volts. The RNA was then visualised using a UV-light transilluminator and a picture was taken. After the gel had been soaked in DEPC-treated water with several changes to dialyse out the formaldehyde, it was treated with 0.05 N NaOH for 20 min at room temperature. The gel was then washed with 20X SSC for 45 min and the RNA was transferred overnight to Hybond-N nylon membranes (Amersham, Buckinghamshire, UK) by capillary blotting in 20X SSC. After transfer, the membrane was washed in 5X SSC and the RNA was crosslinked to the membrane using an UV Stratalinker (Stratagene, California, USA) and kept at room temperature until hybridisation.

The membranes were pre-hybridised and hybridised using the Church's buffer. For Northern and Southern analysis a DNA fragment containing nucleotides 112 to 1084 of the mouse GPI-PLD was used. This 972 bp fragment was obtained by PCR using the GPI-PLD clone 19 DNA as a template and using primers V5' (sense) and V3' (reverse). The
PCR product was gel purified and used as a template for the generation of radioactive probes as described.

2.3.3 RNAse protection assays (RPA)

a) Labelling of probes

High specific activity radioactive antisense RNA probes were synthesised by mixing 0.5 mM of rATP, rUTP and rGTP, 2.5 µl of transcription buffer 5X (Promega, Southampton, UK), 0.25 mM of DTT, 1.5 µg of linearised DNA template, 1.6 U of ribonuclease inhibitor RNasin, 1 µM of rCTP, 5 µl (50 µCi) of \([\alpha^{32}\text{P}]-\text{CTP}\) and 15 U of T7 RNA polymerase. Low specific activity antisense probes (for actin) were synthesised following the same protocol as for high specific activity probes but using only 40 µM rCTP and 0.5 µl (5µCi) of \([\alpha^{32}\text{P}]-\text{CTP}\). The reactions were incubated at 4°C and after 1 h, further 15 U of T7 RNA polymerase were added and the incubation continued for one additional hour.

DNA templates were removed with 20 µg of RNAase-free DNAase (20 mg/ml) for 15 min at 37°C. The RNA probes were precipitated by adding 100 µg of glycogen, 50 µl of Buffer D, 100 µl of isopropanol and incubation at -20°C for 1h. The radioactively labelled probes were centrifuged at 14 000 rpm for 15 min at 4°C, washed with 70% ethanol and dissolved in 1 ml of DEPC-treated water. An aliquot was used to determine the specificity in counts per minute (cpm) of the probe using in a scintillation counter.
b) Hybridisation
10 μg of total RNA were centrifuged at 10 000 g for 10 min at 4°C, washed with 70% ethanol and dissolved in 5 μl of DEPC-treated water. The RNA was mixed with 400 000 (5' end) or 250 000 (3' end) cpm of the GPI-PLD antisense probes and/or 20 000 cpm of the actin probe and 20 μl of RPA hybridisation buffer. Following heat denaturation at 100°C for 3 min, the reaction mixture was immediately placed in an oven at 42°C and hybridised overnight (Figure 2.2).

c) Digestion
The hybridisation was terminated by adding 200 μl of RPA digestion buffer containing 2 μl of Ribonuclease T1 (2 mg/ml) and Ribonuclease A (40 μg/ml) cocktail (Ambion, Abingdon, UK) and incubated 30 min at 37°C. The ribonuclease treatment was stopped by adding 100 μg of glycogen, 300 μl of Buffer D and 500 μl of isopropanol. The solution was precipitated for 1 h at -20°C and then centrifuged at 10 000 g for 15 min at 4°C. The pellet was washed with 70% ethanol, resuspended in 8 μl of loading buffer, denatured 3 min at 100°C and then placed on ice for 2 min. The samples were loaded on a denaturing 8 M urea/6% polyacrylamide gel and run at 1500 volts for 1.5 h. Control samples of undigested probes and yeast RNA were also included in the gel. After complete electrophoresis, the gels were vacuum-dried and exposed to an autoradiograph X-OMAT film at -80°C (Figure 2.2).
Figure 2.2. Overview of the Ribonuclease Protection Assay (RPA). The total RNA samples are incubated overnight at 42°C in the presence of GPI-PLD and actin labelled antisense probes. This incubation allows hybridisation and formation of complementary RNA-probe complexes. In the digestion step, single-stranded and non-complementary sequences are degraded by incubation with a ribonucleases cocktail. The resulting RNA-probe complexes are then precipitated and analysed by PAGE and autoradiography.
2.4 CELL BIOLOGY METHODOLOGY

2.4.1 Cell culture conditions

Cultured cells were kept in an incubator at 37°C in a humidified atmosphere containing 5% CO₂. BHK, 293T, HepG2 (provided by Dr. S. Dawson, UCL, London UK), and COS-7 (provided by Dr. M. Andrawiss, UCL, London, UK) were grown in Dulbecco's modified-Minimum essential medium Eagle (DMEM) and 10% foetal calf serum (FCS). Mouse pancreatic β-cells NIT-1 (ATCC, CRL-2055) were grown and propagated in Ham's F12K medium and 10% heat-inactivated and dialysed FCS. RAW264.7 macrophages were grown in RPMI medium and 10% FCS. All cultures were supplemented with 100 U/ml penicillin, 2 mM L-glutamine, 50 μg/ml streptomycin, 0.1 mM non-essential aminoacids, and 1 mM sodium piruvate.

BHK, 293T HepG2 and COS-7 cells were passaged by rinsing cells with PBS after the medium was removed. After removing the PBS, 3 ml of 0.25% trypsin solution (prepared in versene) was added and the cells were incubated at room temperature (or 37°C) for 2-5 min, until the cells detached. The flask was firmly tapped to dislodge all the cells before additional culture medium was added and the cells harvested by centrifugation. The cells were either dispensed in new flasks at ratios of 1:2-1:10 or transferred to tubes to be washed (centrifugation for 5 min at 1000 rpm and 4°C) and used in further analysis or experiments.

2.4.2 Transfection protocols

a) DEAE-dextran

COS-7 cells were transfected using the DEAE-dextran/chloroquine method (Aruffo, 1997). 2x10⁵ COS-7 cells were plated in 6-well plates the day prior to transfection and grown
overnight in order to obtain ~70% confluence on the next day. On the day of transfection, for each well, 4 ml of DMEM were thoroughly mixed with 80 μl of DEAE-dextran/chloroquine solution, before adding 5 μg of plasmid DNA. Medium was aspirated from the cells and the DNA-containing medium was added to each well. After 3-4 h incubation at 37°C in the CO\textsubscript{2} incubator, DNA-containing medium was removed and replaced with 4 ml of 10% DMSO (in PBS). After 2 min incubation the DMSO was removed and 10 ml of 10% serum-complemented medium were added. The cells were grown overnight before being transferred to new 10-cm Petri dishes.

b) Transfection of BHK, HepG2 and 293T cells

BHK, 293T and HepG2 cells were transfected using the calcium phosphate method slightly modified depending on the cell type (Heinzel et al., 1988; Pear et al., 1993; Jordan et al., 1996). For BHK and HepG2, cells were plated onto 6 well-plates in DMEM at a density of 2x10\textsuperscript{5} cells/well. The cells were allowed to attach overnight. On the day of transfection, DNA precipitates were prepared using room temperature stock solutions: for each plate, 5 μg of plasmid DNA and 31 μl 2M CaCl\textsubscript{2} was mixed with sterile water in a final volume of 250 μl. The DNA mixture was transferred, in a dropwise manner, to another tube containing 250 μl 2X HBS buffer.

After 2 min incubation, the precipitate was pipetted into the media of dish/well, swirled gently to mix and incubated overnight. The next day, the transfected cells were washed with serum-free medium to remove the precipitate, and fresh normal medium complemented with 10% FCS was added. Transfected cells were harvested after 48-72 h and used for subsequent analysis and experiments.
293T cells were transfected in 6-well plates. 4x10^5 cells/well Cells were plated the night before to give 60-70% confluence at the day of transfection. One hour prior transfection, the medium in the wells was replaced with medium containing 25 μM chloroquine and the cells were incubated at 37°C for 1 h.

The DNA precipitate was prepared as follows: 2 μg of DNA was mixed with sterile water in a final volume of 438 μl and 62 μl of 2M CaCl₂ were added. When this DNA solution was ready, it was added in a dropwise manner to 500 μl of 2X HBS buffer while gently mixing. After 2 min incubation, this mixture was added to the cells in a dropwise manner. After 7 h incubation, the cells were rinsed and DMEM medium supplemented with 10% FCS was added. Cells were harvested after 24-72 h for subsequent analysis.

For intracellular staining, 10,000 cells were allowed to attach to circular 13 mm coverslips (BDH, Poole, UK) contained in 4-well plates. For transfection, 1/10 of the amounts used in 6 well plates was used.

2.4.3 Cholesterol depletion and disruption of Golgi complex.

Cells growing on circular coverslips were transfected as described. 24 h post-transfection, cholesterol was depleted with 2% methyl-β-cyclodextrin in DMEM for 1 h following 20 h of serum starvation (Furuchi and Anderson, 1998). For Golgi complex disruption, 48 h post-transfection, cells were incubated with nocodazole (20 μM, dissolved in 1% DMSO) for 1-2 h.
2.4.4 Intracellular staining

Cells were grown and transfected on circular glass coverslips placed on the bottom of 4 well plates. After 24-48 h of transfection, cells were washed four times with 250 μl PBS and fixed for 15 min at room temperature with a 4% PFA solution (freshly prepared in PBS). Cells were rinsed 6 times with PBS before being permeabilised with a 0.2% Triton X-100 solution for 5 min. Detergent was eliminated with four washes with PBS.

Cells were blocked with a 10% rabbit serum solution for 15 min. After incubation, the blocking solution was removed and 250 μl of the primary antibody solution (resuspended in PBS containing 0.2% BSA) was added to the cells without washing. The cells were incubated at room temperature for 1 h in the presence of primary antibody and then washed 6 times. The secondary antibody (resuspended in PBS containing 0.2% BSA) was added and the cells were incubated for 45 min before 6 washings with PBS. Control experiments to show the specificity of the labelling included non-specific antibodies of the same isotype to that of primary antibodies. The circular coverslips were mounted onto slides using 7 μl of Mowiol mounting medium. Slides were left overnight at 4°C before being analysed in a confocal microscope.

2.4.5 Confocal microscopy analysis

Cells were analysed by confocal microscopy with a Bio-Rad MRC 1024 confocal system (Bio Rad Laboratories, Hertfordshire, UK) equipped with an argon and helium/neon laser for excitation at 522 and 585 nm. The system is attached to a Zeiss Axiovert 100TV inverted microscope with 10x, 20x, 40x and 63x objectives. Images were acquired with the LaserSharp 3.2 software. Phycoerythrin and GFP or FITC fluorescence were recorded sequentially using a 40X objective. For overlay, images were adjusted to the same output
intensities and merged with the Confocal Assistant 4.2 program into a composite RGB image. Figures were arranged with Microsoft Power Point.

2.4.6 Quantification of TNFα production

RAW264.7 (80 000 cells/well) macrophages were seeded in flat bottom 96-well plates in a final volume of 200 μl and left to adhere for 2 h at 37°C before the non-adherent cells were removed. Cells were incubated for 6 h in 200 μl RPMI medium containing either 10 ng/ml LPS, 100 nM VIP or a combination of these two molecules. TNFα production was assayed using a standard sandwich enzyme-linked immunosorbent assay (ELISA).
2.5 BIOCHEMICAL METHODOLOGY

2.5.1 Sucrose gradients

Membranes of transfected BHK cells were fractionated using a detergent-free procedure according to Song et al., (Song et al., 1996). Basically, after two washes with cold PBS, three confluent 10-cm dishes of transfected BHK cells were harvested following trypsin/versene treatment and centrifugation. The cells were resuspended in 1.66 ml of 500 mM sodium carbonate (pH 11) and disrupted by ten strokes in a loose-fitting homogeneiser (Jencons, Bedfordshire, UK) and three bursts (20 sec, 1.8 Amp) in a Ultrasonic Power unit (MSE, York, UK). The homogenates were adjusted to 45% sucrose by the addition of 90% sucrose prepared in MBS buffer, and placed at the bottom of an ultracentrifuge tube.

A 5-35% discontinuous sucrose gradient was made with 3.32 ml of 35% sucrose overlaid with 3.32 ml of 5% sucrose; both in MBS containing 250 mM sodium carbonate and centrifuged in a SW41 rotor at 35 000 rpm for 20 h at 4°C in a L7-65 ultracentrifuge (Beckman, California, USA). Ten 1 ml fractions were collected from the top of the gradient. 37.5 μl of each fraction were mixed with 12.5 μl of 4x SDS-PAGE running buffer, boiled for 3 min, resolved by SDS-PAGE, and analysed by Western Blot.

2.5.2 Reducing SDS-polyacrylamide gel electrophoresis (PAGE)

For electrophoresis the method described by Laemmli, in which denatured and reduced proteins are separated at a high voltage over a pH gradient in a poly-acrylamide gel was used (Laemmli, 1970). Small (8x6 cm) or big (16x11cm) 10-12.5 % polyacrylamide resolving gels and 5% polyacrylamide stacking gels were made. Protein samples and Rainbow-coloured protein markers (Amersham, Buckinghamshire, UK), were mixed with
2X SDS-PAGE loading buffer, boiled for 5 min and loaded on gels. Gels were run using SDS-PAGE running buffer at a constant 100V until the bromophenol blue dye had run out of the bottom of the resolving gel.

### 2.5.3 Western blot

Proteins were electrophoretically transferred from acrylamide gels to transfer buffer-soaked HyBond C extra nitrocellulose membranes (Amersham, Buckinghamshire, UK) overnight at a constant 30 V (big gels) or for 3 h at a constant 70 V (small gels) using either Gibco V15-17 tanks and a Transblot Cell (Gibco, Paisley, UK) or a Miniprotean 3 Cell System (Bio-Rad, Hertfordshire, UK). The membranes were blocked for 1 h with continuous shaking in 2% Marvel solution prepared in TBS containing 0.1% Tween 20 (TBS-T).

Primary antibodies were diluted in blocking solution and incubated with the membranes in sealed plastic bags for 1 h with continuous shaking at room temperature. Membranes were washed three times with TBS-T for 10 min each time and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in blocking solution for 1 h with continuous shaking. Following three washes with TBS-T proteins were detected by using an enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, UK) and Biomax MR films (Kodak, New York, USA).

### 2.5.4 ELISA

TNFα production by stimulated macrophages was evaluated by a standard sandwich ELISA as follows: 96-well plates were coated overnight at 4°C with 50 µl/well of hamster monoclonal anti-mouse TNFα antibody solution (1:1000) prepared in carbonates buffer.
Wells were then washed four times with PBS-Tween 20 and blocked at 37°C for 1 h with 200 μl of 2% Marvel in carbonates buffer. After four washes with PBS-Tween 20, 100 μl of supernatants from stimulated macrophages were added followed by incubation at room temperature for 2 h. Plates were washed and further incubated at room temperature for 1 h with 100 μl of a rabbit anti-mouse TNFα antibody solution (1:3000) prepared in PBS-Tween 20 containing 3% bovine serum albumin (BSA). This was washed and incubated with a goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugated antibody solution (1:3000, 100 μl/well) prepared in PBS-Tween 20 containing 3% BSA. After final washings and addition of 100 μl of HRP substrate solution, plates were incubated at room temperature for 15 min and read at 450 nm in a MRX TC Revelation plate reader (Dynex Technologies, VA, USA).

2.5.5 Isolation of [3H]-myristate-labelled membrane-form VSG from trypanosomes.

a) Preparation of trypanosomes

F1 (BALB/cxCBA/Ca) mice weighing 20-25 g were infected intraperitoneally with stabilate of *Trypanosoma brucei* strain 221 (kindly done by Dr. David Horn, London School of Hygiene and Tropical Medicine, UK) with 5x10⁵ or 1x10⁶ trypanosomes. The course of the infection was assessed by estimating the number of trypanosomes from tail bleeds. When parasitaemia reached 1-5x10⁹/ml (usually after three days of infection) mice were exanguinated. The blood was diluted 1 in 4 with PSG buffer, containing 10 U/ml heparin and kept on ice.
b) Isolation of bloodstream trypanosomes

Trypanosomes were isolated by DEAE-cellulose chromatography (Lanham et al., 1970). 0.5 kg of DE52 DEAE-cellulose (Whatman, Maidstone, UK) was suspended in 1.25 l of PSG buffer and the resulting slurry was adjusted to pH 8.0 with orthophosphoric acid (1:20 v/v dilution). The slurry was mixed with a magnetic stirrer for 30 min and then allowed to stand without stirring for 15 min. The supernatant was discarded and the cellulose was subjected to a further 6 washes. The slurry was stored at 4°C.

To prepare the isolation column, a Whatman No. 41 filter paper was moistened with PSG buffer and placed on the plate of a Büchner funnel. Approximately 50 ml of the slurry containing the DEAE-cellulose was poured into the funnel and excess liquid was run out gently through the column, allowing the cellulose to form a firm horizontal surface. Another moistened Whatman filter paper was placed on the cellulose bed.

The cold diluted blood was carefully layered on to the surface. When all the blood had entered the column, trypanosomes were eluted with 6 columns-volume of PSG and collected in a flask and kept on ice. The fractionated trypanosomes were washed once with 20 ml of ice-cold PSG and centrifuged at 220 g for 10 min at 4°C. The yield of trypanosomes was enumerated using a haemocytometer.

c) \[^{3}H\] myristate labelling of bloodstream trypanosomes

The labelling was based in the method described by Ferguson (Ferguson and Cross, 1984). 1 mCi of \[9,10(n)^{-3}H\] myristic acid (specific activity 54.0 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK) was dried, redissolved in 5 μl of 95% ethanol, and mixed with equimolar fatty acid-free BSA (20 mg/ml in H₂O). 2×10⁹ trypanosomes were suspended in 10 ml of VSG labelling medium, and incubated at 37°C for 15 min.
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After this initial incubation period, [9,10(n)-\(^3\)H] myristic acid was added at 100 \(\mu\)Ci/ml and trypanosomes were incubated at 37°C for 1 h.

d) Isolation of \(^3\)H myristate-labelled membrane form-VSG

\([^{3}\text{H}]\) myristate-labelled VSG was isolated according the protocol described by Hereld et al. The labelled trypanosomes were cooled to 0°C and centrifuged at 3 000 g, 10 min, 4°C in a J-6B Beckman centrifuge. The supernatant was saved for subsequent labelling. The cell pellet was washed with 5 ml of ice-cold PSG and centrifuged for 10 min at 3 000 rpm and 4°C. The supernatant was discarded (Hereld et al., 1986).

The trypanosomes were lysed osmotically in 10 ml of VSG lysis solution. After 10 min on ice, the lysate was centrifuged for 5 min at 5 000 rpm and 4°C, and the pellet was washed in 4 ml of the same buffer. The washed pellet, which contained the membrane-form (mf) VSG, was extracted with 8 ml of CHCl\(_3\)/CH\(_3\)OH (2:1) at 20°C and centrifuged 13 000 g for 5 min in a J2-21 centrifuge. The pellet was dissolved in 4 ml of 1% SDS by heating at 100°C for 10 min and vortexing. To remove remaining \(^3\)H-labelled lipids, the solution was extracted twice with 6 ml of n-butyl alcohol at 20°C. For each extraction, the solution was vigorously homogenised and centrifuged at 13 000 g for 5 min to separate the two phases. In the third extraction, 9 ml of water-saturated n-butyl alcohol was used to maintain the aqueous phase. A fourth extraction, using 9 ml of n-butyl alcohol, eliminated the aqueous phase and produced a gummy precipitate.

This precipitate was recovered by centrifugation at 13 000 g for 5 min at 4°C, washed with anhydrous ether, and air-dried before being dissolved in 1 ml of 1% SDS by heating at 100°C for 10 min. Insoluble materials were removed by centrifugation at 13 000 g for 5 min at 20°C. The product was assayed by SDS-PAGE and Coomassie staining.
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2.5.6 GPI-degradation assay

GPI degradation by cells transfected with GPI-PLD-encoding plasmids was monitored by using [³H] myristate-labelled VSG as substrate (Hereld et al., 1986). Transfected cells (2x10⁵) were harvested and washed with cold PBS. The cell pellets were resuspended in 200 µl VSG buffer A containing 0.5% Nonidet P40 (NP-40) and 1X cocktail of protease inhibitors. After vigorous vortex mixing and incubation on ice for 30 min, the nuclei were removed by centrifugation at 4°C for 5 min at 14 000 rpm in a microfuge, and the supernatant assayed for GPI-degrading activity.

For the degradation assay, 10 µl of the NP-40 cellular extracts and 90 µl of VSG buffer A were mixed in eppendorf tubes. The reaction was started by adding 0.1 ml of VSG substrate mixture, and incubating at 37°C for 60 min. The reaction was stopped by the addition of 0.5 ml of 1 M NH₄OH-saturated butanol. After 2 min of vortex mixing, phase separation was achieved by 3 min centrifugation in a microcentrifuge. 0.3 ml of the upper organic phase was sampled, mixed with 5 ml of scintillation fluid and counted by liquid-scintillation.

2.6.7 in vivo assay for GPI-PLD activity

The in vivo activity of GPI-PLD was evaluated by the release of placental alkaline phosphatase (PLAP) in COS-7 and 293T cells co-transfected with plasmids encoding for GPI-PLD and a GPI-anchored form of PLAP (Kung et al., 1997). Cells grown in alkali-treated serum were co-transfected in 6-well plates and after 48-72 h supernatants and cells were harvested. Cells were lysed by sonication in 200 µl of 50 mM MES buffer (pH 6.5). Aliquots of sonicated cells (5 µl) or supernatants (50 µl) were mixed with 150 µl of PLAP assay solution and incubated in a final volume of 200 µl at room temperature.
Conversion of $p$-nitrophenylphosphate to $p$-nitrophenol, by the PLAP present in supernatants or cell extracts, was measured spectrophotometrically at 405 nm over a period of 5-15 min in a MRX Tc ELISA plate reader (Dynex Technologies, CA, USA).
CHAPTER 3

ANALYSIS OF THE MOUSE LIVER GPI-PLD GENE

3.1 INTRODUCTION

Over the past years, many different cell surface proteins that use the glycosylphosphatidylinositol (GPI) moiety as a membrane anchor have been described. The GPI-anchored proteins constitute a growing family of functionally diverse proteins which have been identified in several systems ranging from protozoa and yeast to mammalian cell lines (Gerold et al., 1996).

GPI-anchored proteins can be released from the cell surface by different and well-characterised phospholipases derived from bacteria. These phospholipases, however, do not cleave acylated GPI-molecules and are not specific for the GPI-anchors because they can also recognise and cleave phosphatidylinositol (PI) structures (Griffith et al., 1991).

GPI-specific phospholipase C (GPI-PLC) isolated from trypanosomes has been described and characterised (Bulow et al., 1989). Mammalian GPI-PLC activities have been reported (Fox et al., 1987; Fouchier et al., 1990; Stieger et al., 1991) and several studies on adipocytes and porcine proximal tubules suggest that this enzyme has an important role in the release of GPI-anchored proteins (Movahedi and Hooper, 1997; Park et al., 2001). However, the cloning and complete characterisation of these phospholipases has not yet been completed. So far the only phospholipase with specificity exclusively for GPI moieties cloned from mammals is GPI-phospholipase D (GPI-PLD), which has been purified from bovine and human serum (Davitz et al., 1987; Metz et al., 1991).
By the time this work was initiated, GPI-PLD had been cloned from different sources and three different GPI-PLD cDNA sequences had been reported: one from bovine liver (GeneBank accession number M60804), one from human pancreas (GeneBank accession number L11702) and one from human liver (GeneBank accession number L11701), all showing a high degree (84-95%) of homology (Scallon et al., 1991; Tsang et al., 1992).

Analysis and comparison of the human liver and pancreas GPI-PLD DNA sequences revealed some nucleotide differences that resulted in changes in the predicted amino acid sequences. When liver and pancreas GPI-PLD protein sequences were compared, 42 amino acid differences scattered all along the sequences were found (LeBoeuf et al., 1998). In addition, Northern blot analysis of total bovine liver RNA demonstrated the expression of two distinct GPI-PLD mRNAs (Stadelmann et al., 1993).

It is possible that the differences found in the two human sequences could be the result of genetic polymorphisms, which suggest that at least in humans, two or more genes encode for GPI-PLD (Tsang et al., 1992). However, it is also possible that the detection of two distinct GPI-PLD mRNAs in bovine liver can be explained by alternative splicing of mRNA derived from a single GPI-PLD gene (Stadelmann et al., 1993).

The GPI-PLD primary structure has been deduced from a full-length cDNA construct obtained from a bovine liver cDNA library. The predicted polypeptide product contains a 23-amino acid signal peptide and a translation stop codon at the 3' end suggesting that the GPI-PLD gene encodes a mature protein of 817 amino acids with a molecular weight of ~90 kDa (Scallon et al., 1991). The deduced amino acid sequence revealed four regions of internal homology (amino acids 357 to 379, 426 to 448, 489 to 511 and 694 to 716) with a high degree of similarity to metal-ion (Ca\(^{2+}\) and Mg\(^{2+}\)) binding domains in the alpha subunits of proteins belonging to the integrin family. Further
sequence analysis revealed multiple potential sites of N-linked glycosylation and phosphorylation (Huang et al., 1990; Scallon et al., 1991; Hynes, 1992; Springer, 1997).

GPI-PLD is relatively abundant in serum, but it is not clear which organ is the source of this enzyme. Northern blot analysis suggest that liver is the major source of GPI-PLD (LeBoeuf et al., 1998; Schofield and Rademacher, 2000), and supporting this, it has been found that its concentration is dramatically reduced in liver-damaged patients (Raymond et al., 1994; Maguire and Gossner, 1995; Tujoaka et al., 1998). There is also evidence indicating that apart from the liver other organs also contribute to circulating GPI-PLD. However, the biological significance of high concentrations of circulating GPI-PLD has not been established (Hoener et al., 1990; Metz et al., 1991; Stadelmann et al., 1993; Deeg and Verchere, 1997; Rhode et al., 1999).

Despite the potential roles of GPI-PLD in regulating the expression of GPI-anchored proteins and the generation of second messengers for growth factors such as insulin (Rademacher et al., 1994; Jones et al., 1997; Jones and Varela-Nieto, 1998; Low and Prasad, 1988), many questions about the sites and mechanisms of action and their regulation remain unanswered. An important question relates to the identity of the specific cells and tissues that produce GPI-PLD and whether more than one gene codes for this enzyme. It could be possible that the expression of different GPI-PLD isoforms or alterations in the expression of this enzyme could be associated with pathological conditions.

The experiments described in this chapter were undertaken with two major aims. First, to establish whether one or more genes encode for mouse GPI-PLD, and secondly to analyse the expression of GPI-PLD in tissues from normal, diabetic and diabetic susceptible mouse strains.
3.2 RESULTS

3.2.1 Screening of a mouse liver cDNA library.

A mouse liver cDNA library consisting of $5 \times 10^5$ clones inserted into the Sal I/Not I sites of the pSport 1 plasmid was obtained from the German Human Genome Project (DHGP). This library was screened with a GPI-PLD specific rat cDNA probe (provided by Dr. J. Schofield, Department of Immunology and Molecular Pathology, UCL, London) and three different cDNAs that hybridised to the GPI-PLD probe were isolated. The three different clones (9, 12 and 19) had molecular sizes of 1440, 1200 and 3200 bp, respectively (see Figure 3.1).

**Figure 3.1.** Mouse GPI-PLD liver DNA clones. cDNA clones 9, 12 and 19 were isolated from a liver cDNA library by screening with a rat GPI-PLD probe. Digestion of 5 μg of each pSPORT1-GPI-PLD clones with Sal I and Not I enzymes produces DNA fragments of 1400, 1200 and 3200 bp for clones 9, 12 and 19, respectively. Digestion products were resolved in a 1% TAE-agarose gel.
3.2.2 Sequencing analysis of the mouse GPI-PLD clones

The different molecular sizes of clones 9, 12 and 19 suggested, either that the GPI-PLD insert might be derived from different genes, were the product of alternative splicing events or could represent truncated forms of the same gene. To address this question the 5' and 3' ends of the GPI-PLD clones were sequenced. This showed that:

- Clones 9 and 12 have 3' ends homologous to the 3' untranslated region of GPI-PLD and they have 5' ends with homology to regions of the middle part of the bovine GPI-PLD. Thus it is unlikely that these two clones were the products of short isoforms of the GPI-PLD gene. They are more likely truncated cDNA clones obtained as cloning artefacts during the construction of the library.

- Clone 19 has 5' and 3' ends homologous to the 5' and 3' non-translated regions of the GPI-PLD. Because of the molecular size of this clone (3200 bp), it most likely represents a nearly full-length transcript containing the entire coding region. With these preliminary sequencing results in mind, subsequent analysis was done using clone 19.

Shortly after the partial sequencing analysis of the GPI-PLD clones was initiated, a mouse GPI-PLD cDNA sequence isolated from a pancreatic cell line was published (LeBoeuf et al., 1998). Preliminary sequencing data from clone 19 showed some nucleotide sequence differences when compared with the published sequence, therefore it was decided to determine the entire DNA sequence of clone 19. For this, a Genome Priming System (GPS) was used to generate templates for the DNA sequencing.

The GPS is a Tn7 transposon-based in vitro system that uses TNsABC* transposase to randomly insert a transposon into a target DNA (Stellwagen and Craig, 1997a;b). The transposon contains an antibiotic-resistance marker and two unique primer sequences, which allowed sequencing of the GPI-PLD clone 19 in both orientations. After
transformation of XL1-Blue competent cells and selection with ampicillin and chloramphenicol, 28 colonies were selected and the plasmid DNA extracted. Eleven plasmids (indicated by arrows in Figure 3.2) produced fragments of about 4000 bp when digested with SalI and NotI restriction enzymes, indicating that the transposon had integrated into the GPI-PLD sequence. These were selected and sequenced using the N and S primers provided with the GPS kit.

Figure 3.2. Integration of transposons into the GPI-PLD clone 19. GPS transposon-donor plasmid and pSPORT1-GPI-PLD clone 19 plasmid were incubated in the presence of transposase. New vectors with inserted transposons were used to transform XL1-Blue bacteria. After selection with ampicillin and chloramphenicol, 28 antibiotic-resistant colonies were grown and plasmid DNA extracted. 5 μg of plasmid DNA with integrated transposons was digested with SalI and NotI restriction enzymes. Those templates producing fragments of about 4000 bp (indicated by arrows) were selected for automated sequencing.

Transposons were inserted into the GPI-PLD sequence in only 8 out of the 11 selected templates. In the other three templates the transposons were inserted into the pSPORT1 plasmid. The DNA sequences obtained from these 11 plasmids were analysed and aligned with a Contig Assembly Program (CAP) (Smith et al., 1996) to obtain a 3203-bp consensus sequence (Figure 3.3). This sequence has been
based_in_the_National_Center_for_Biotechnology_Information_(NCBI)_nucleotide_database_under_the_accession_number_AY081194.

Based_on_the_reported_bovine_sequence_(Scallon_etal.,_1991),_GPI-PLD_clone_19_contains_an_open_reading_frame_of_2526_bp_counting_from_the_second_methionine_codon,_a_43-bp_5'_untranslated_region,_and_a_631-bp_3'_untranslated_region,_which_contains_a_stop_codon_and_a_polyadenylation_signal_(AATAAA).

The_published_GPI-PLD_sequencederived_from_mouse_pancreatic_cells_(LeBoeuf_etal.,_1998)_and_the_GPI-PLD_clone_19_consensus_sequence_showed_a_high_degree_of_homology_albeit_with_some_differences:
- 9 single_base_differences_between_the_two_sequences_that_changed_the_amino_acid_sequence_in_4_codons.
- One_insertion_of_twelve_bases_(452-464) in_the_5'_end, and_one_insertion_of_six_bases_in_the_middle_part_(1352-1358) of_the_liver_consensus_sequence.
- One_deletion_of_three_bases_in_the_liver_consensus_sequence_(1050-1052).
- Three_separate_single_base_insertions_in_the_3' end_of_the_consensus_sequence.
- 227_nucleotides_in_the_untranslated_5' end_of_the_pancreatic_sequence_not_present_in_the_liver_sequence.
- 24_nucleotides_in_the_untranslated_3' end_of_the_pancreatic_sequence_not_present_in_the_liver_sequence.
Figure 3.3. Alignment of mouse pancreatic GPI-PLD DNA sequence (P GPI-PLD) and the liver consensus sequence obtained in this thesis (L GPI-PLD). Differences between the two sequences are shown in bold red letters. Bold blue letters indicate the initiation and stop codons. Bold green letters indicate the polyadenylation signal in the 3' end.
CHAPTER 3. ANALYSIS OF THE MOUSE LIVER GPI-PLD GENE.
3.2.3 Translated polypeptide sequence

GPI-PLD clone 19 consensus sequence codes for a protein of 842 amino acids. Although the differences between the mouse pancreas- and liver-derived DNA sequences did not change the reading frame, they altered the amino acid sequence. When the predicted amino acid sequence for clone 19 was aligned with the pancreas-derived GPI-PLD protein sequence (Figure 3.4), some differences were found:

- Five single amino acid differences between the two sequences: residues 130 (L-V), 134 (N-S), 541 (R-G), 544 (K-R) and 829 (R-W).
- Two insertions of four (residues 136-139) and two (residues 437-438) amino acids, respectively in the liver consensus sequence (corresponding to the 12 and 6-nucleotides insertions in the DNA sequence).
- One single amino acid deletion (residue 335) in the liver consensus sequence (corresponding to the three-nucleotide deletion in the DNA sequence).

With exception of the residue at position 544, where a lysine residue is conserved in the human, bovine and mouse pancreas-derived sequences, all the amino acid differences found in the mouse liver-derived sequence are shared by the two human and the bovine sequences (see Figure 3.5).

The regions with homology to the Ca^{2+}-binding domains of the α-subunit of integrins, previously described in the bovine GPI-PLD (Scallon et al., 1991) were not affected by the amino acid changes described above (see Figures 3.4 and 3.6). A search for protein motifs in the GPI-PLD clone 19 sequence, identified the sequence FLVEQFQDY (residues 238-246) which corresponds to the motif \( \Psi^{XXXX}\Psi^{XX}\Psi \) (where \( \Psi \) represents an aromatic amino acid and \( X \), any other amino acid) recognised by caveolins (Couet et al., 1997b) (Figure 3.4). Because GPI-PLD and caveolin are situated in opposite sides of the membrane, the biological significance of this caveolin recognition site has yet to be established, since it is not known whether GPI-PLD localises to caveolae or more specifically can interact with caveolins.
## Figure 3.4. Alignment of GPI-PLD protein sequences derived from mouse pancreas (PGPI-PLD) and mouse liver (LGPI-PLD). Amino acid differences between the two sequences are shown with red letters. The sequence motif \(\Psi^{XXXXYXXY}\), where \(\Psi\) represents an aromatic amino acid and \(X\), any other amino acid (recognised by caveolins) is shown in bold blue letters. The regions of internal homology in GPI-PLD to each other and to the metal ion binding domains of alpha subunits of integrins are shown in bold italics. The 23 N-terminal amino acids that predict the signal peptide are underlined.
Figure 3.5. Alignment of GPI-PLD amino acid sequences. Protein sequences derived from human pancreas (HP), human liver (HL), bovine liver (BL), mouse liver (ML, corresponding to the consensus sequence obtained in this work), and mouse pancreas (MP). The differences between the two mouse sequences (indicated by red rectangles) are shown and compared with the human and bovine available sequences. Completely conserved residues are shown in green boxes, identical residues in yellow boxes, similar residues in light blue boxes, and different residues in white boxes, respectively. The N-terminal amino acids corresponding to the signal sequence are indicated in a dark blue rectangle. Sequence alignment was done using the Clustal V software program (Thompson et al., 1994). The color-coded plot of pre-aligned sequences was made using the version 3.3.1 of the BOXSHADE software program.

Further analysis and search for protein motifs within the GPI-PLD consensus sequence revealed different potential sites for protein modification such as O- and N-glycosylation, and phosphorylation by different kinases (Table 3.1) (Hansen et al., 1988; Bucher, 1994). It should be noted that these potential motifs might not represent actual sites for protein modification. For instance, it has been shown that human GPI-PLD sequence has potential sites for tyrosine phosphorylation, however native GPI-PLD was not phosphorylated when using a partially purified tyrosine kinase.
kinase from T lymphocytes. In contrast, proteolytic fragments of GPI-PLD were phosphorylated suggesting that the tyrosine phosphorylation sites may not be accessible in the native form (Civenni et al., 1999). It should be also noted that despite GPI-PLD contains a caveolin-binding motif, no sites for lipid modification such as myristoylation were found in the motif search. This lipid-modification is an important characteristic shared by proteins interacting with caveolin (Okamoto et al., 1998).

<table>
<thead>
<tr>
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<th>N-glycosilation</th>
<th>cAMP-PKA site</th>
<th>PKC-site</th>
<th>CK2-site</th>
<th>Tyrosine kinase sites</th>
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</table>

Table 3.1. Potential protein modification sites in GPI-PLD. The GPI-PLD protein sequence was obtained from translation of clone 19 GPI-PLD DNA consensus sequence. Search for motifs was done by using software and databases in the PROSITE web site (Bucher, 1994).
3.2.4 Comparison of GPI-PLD clone 19 consensus sequence to expressed sequence tags (EST) clones.

Possible explanations for the DNA sequence differences between GPI-PLD clone 19 and the pancreas-derived sequence are:

- More than one gene codes for mouse GPI-PLD.
- The differences are due to the expression of alternative spliced forms or there is a tissue-specific/mouse-strain specific expression.
- All these differences are possibly due to sequencing errors either in the published sequence or in the sequence of clone 19.

To address these alternative explanations, the sequence of the mouse liver and pancreas-derived GPI-PLD sequences were compared to expressed sequence tags (EST) deposited in GeneBank. EST sequences have been obtained by sequencing randomly picked cDNA clones from different cDNA libraries as part of the Human Genome Project. In Table 3.2 a list of EST clones with homology to the certain regions of GPI-PLD clone 19 consensus sequence is shown. Different EST sequences were found that showed identity with the liver sequence rather than the pancreatic sequence.

For instance, the deletion of three nucleotides in the middle part of the liver GPI-PLD sequence (nucleotides 1050-1052) is present in the EST clones BF531809 and BI149176 (derived from FVB/N mouse liver), AI527199 and BB579855 (derived from C57BL/10 mouse liver). See Figure 3.7 for the alignment of these sequences. In addition, the insertion of twelve nucleotides in the 5' end of the liver GPI-PLD (nucleotides 452-464) sequence is also present in the EST clones BF660153 and AI313997 (from house mouse liver and from C57BL/10 mouse liver, respectively), BG085375 and BB586843 (from mouse embryonic and bladder tissues, respectively). See Figure 3.7.
<table>
<thead>
<tr>
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<th>EST clone</th>
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<td>87 BB185743</td>
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<td>(Hypothalamus)</td>
<td>90 AV274713</td>
<td>C57BL/6J (Testis)</td>
</tr>
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</tr>
<tr>
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<td>FVB/N (Liver)</td>
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</table>

Table 3.2. Expressed sequence tags (EST) clones with homology to GPI-PLD clone 19 consensus sequence. GPI-PLD clone 19 DNA consensus sequence was compared to EST sequences deposited in the NCBI database. Homology between GPI-PLD clone 19 sequence and each EST clone was determined by analysis with the Pairwise Blast program (Tatusova et al., 1999).
Some of the other single nucleotide differences observed between the pancreas and the liver-derived sequences are also present in different EST clones, suggesting that at least the consensus sequence of GPI-PLD clone 19 is correct and that the transcript is present in different tissues. However, as most of the EST clones are derived from mouse liver (Table 3.2), this does not rule out that different transcripts with different sequences may exist in the mouse. Unfortunately, no EST clones derived from pancreatic tissue have been reported so far.

**Figure 3.7.** Alignment of GPI-PLD sequences derived from pancreas, liver and EST clones. Fragments of the pancreas-derived (PGPI-PLD) and the GPI-PLD clone 19 consensus sequence (LGPI-PLD) sequences were compared to DNA sequence fragments derived from EST clones and the sequence differences are shown in red colour. A) Four different EST clones sharing the three-nucleotide deletion detected in the middle part (nucleotides 1050-1052) of LGPI-PLD are shown. B) Four different EST clones sharing the insertion of 12 nucleotides in the 5' end of the liver GPI-PLD sequence (nucleotides 452-464) are shown. Single nucleotide differences found in the EST clones are shown in red. All the complete DNA sequences were aligned by using the Multiple Sequence Alignment ClustalW program (Thompson et al., 1994).
3.2.5 Southern Blot Assays.

As previously indicated, the sequence differences found between the pancreatic and liver-derived GPI-PLD could be the result of more than one gene encoding for the mouse GPI-PLD. In order to evaluate this possibility genomic DNA from CBA/Ca mice was digested with different restriction enzymes and analysed by Southern blot. Digested DNA was transferred to a membrane and hybridised with a 972-bp PCR probe corresponding to nucleotides 112-1084. The results obtained (Figure 3.8) showed the presence of several fragments, which cross-hybridised to the GPI-PLD probe with different degrees of intensity. There are two possible explanations for the many specifically hybridising bands. They could either be derived from one single gene which has large non-coding intron sequences. Alternatively, there could be more than one gene encoding the GPI-PLD sequences. The human genome encodes only one gene consisting of 25 exons separated by very large introns and with exons 1 to 24 ranging from 27 to 208 bp, whereas the final exon 25 is 3224 bp (Schofield and Rademacher, 2000). Since homologous human and mouse genes usually have the same structural organisation one can assume that the mouse GPI-PLD gene has the same structure. The hybridisation result shown in Figure 3.8 is consistent with such a gene organisation, where only a limited number of exons have a sufficient size to hybridise to the radioactive probe. The hybridisation pattern does not exclude the possibility that the mouse genome only contains a single gene encoding GPI-PLD. However, scanning of the mouse genome recently made available, shows only one sequence located on chromosome 13.
Figure 3.8. Southern blot analysis of liver genomic DNA from CBA/Ca mice. 10 μg of liver DNA was digested with restriction enzymes as indicated. DNA fragments were separated by electrophoresis and then transferred to a Hybond N+ nylon membrane. The membrane was hybridised with a 5’ end PCR product, nucleotides 112-1084, of the GPI-PLD. The GPI-PLD contains an internal Bam HI site at position 1359. An arrow points to the strongest band hybridising with the probe in the Bam HI-digested genomic DNA.
3.2.6 Expression of GPI-PLD

From the Southern blot results and assuming that only one gene codes for the mouse GPI-PLD, then the sequence differences observed between the pancreas and liver-derived mouse sequences are likely to be the result of the expression of different transcripts. To evaluate this, the GPI-PLD RNA expression was analysed by Northern blot. Total RNA derived from different CBA/Ca mouse tissues (Figure 3.9) shows the presence of only one major transcript, most likely a full length transcript, of around 4 kb in liver. Less intense bands corresponding to this transcript were also detected in brain, antrum and lung, but not in the other seven tissues tested. The GPI-PLD transcript expressed in brain has a slightly higher molecular size than the transcript expressed in liver.

3.2.7 Ribonuclease protection Assays

The results obtained from the Southern and Northern blots are consistent with the assumption that there is only one gene coding for the mouse GPI-PLD (Figures 3.8 and 3.9). Apart from the possibility of sequencing errors, the possibility that different isoforms of the mouse GPI-PLD gene may exist and be expressed in a tissue-specific manner could explain the differences between the pancreas and liver-derived GPI-PLD sequences. These possibilities could be addressed with the ribonuclease protection assays (RPAs). RPAs are used for detection and quantitation of RNA in a sequence specific manner. The method is based on the ability of ribonucleases to specifically degrade single-stranded RNA while leaving intact the double-stranded RNA homoduplex between a labelled antisense RNA probe hybridised to its mRNA target (Winter et al., 1985).
Figure 3.9. Analysis of the expression of GPI-PLD mRNA in different tissues obtained from CBA/Ca mice. 40 μg of total RNA were loaded in each lane and separated in a formaldehyde-agarose gel. The separated RNA species were transferred to a nylon membrane and hybridised with a PCR product corresponding to the 5' end of the GPI-PLD gene (A). The schematic drawing shows the relative size of the GPI-PLD probe used to hybridise the membrane (nucleotides 112-1084). A picture of the ribosomal 28S RNA stained with ethidium bromide is shown as an indication of total RNA loading (B).
By using a high specific-activity antisense probe, RPAs are sensitive enough to detect single base pair differences between the probe and its target RNA molecule. RPAs are therefore suitable to distinguish between sequence-homologous and distinct transcripts that co-migrate on Northern blots (Prediger, 2001).

3.2.7a Design and synthesis of antisense probes.

Three different GPI-PLD antisense probes were used in the RPA experiments. A flow diagram for the construction of the plasmids used to synthesise the GPI-PLD RNA antisense probes is shown in Figure 3.10.

The 5' GPI-PLD antisense probe was derived from digestion of the GPI-PLD clone 19 with the \textit{Pst I/Cla I} restriction enzymes. The 434-bp fragment (from nucleotide 52 to 486) was subcloned into the \textit{Pst I/Cla I} sites of the pBluescript SK\textsuperscript{*} vector. This fragment contains the 12-nucleotide insertion observed in the liver sequence. The antisense probe was synthesised after digestion of the plasmid with \textit{Pvu II} (see Figure 3.11A).

The antisense probe for the middle part of the GPI-PLD was generated by cloning a 384 bp fragment into the \textit{Srf I} site of the pCRScript SK\textsuperscript{*} vector. This fragment (from nucleotides 1009 to 1393) was obtained by PCR using the primers Sac35 and Sal33 and GPI-PLD clone 19 as template. The fragment covers the region where the 3-nucleotide deletion and the 6-nucleotide insertion were detected in the liver sequence (Figure 3.11B). The antisense probe was synthesised after digestion with \textit{Pvu I} and \textit{Sac I}.
Figure 3.10. Flow diagram for the construction of the plasmids used to synthesise the GPI-PLD antisense probes.
An antisense probe for the 3’ end of the GPI-PLD was generated by cloning a 278-bp fragment into the Bam HI/Sal I sites of the pCRScript SK+ vector. This fragment (from nucleotide 2713 to 2994) was obtained by PCR using the primers 5’BamHI and 3’Sall and GPI-PLD clone 19 as template. This fragment contains three inserted nucleotides detected in the untranslated region of GPI-PLD and the antisense probe was synthesised after digestion with Pvu II (Figure 3.11C). All three antisense probes were synthesised by using T7 RNA polymerase.

The pTRI-β-Actin-Mouse plasmid (Ambion, Oxfordshire, UK) containing a 250-bp fragment of the mouse cytoplasm actin (β-actin) gene was used to generate actin antisense probes. When transcribed with T7 RNA polymerase, the Hind III-linearised plasmid produced a 335-bp antisense probe that generated a 250-bp protected fragment. In some experiments, the pTRG-Actin-Mouse plasmid was digested with Hind III and Dde I to produce a 200-bp antisense probe with a 150-bp protected fragment.

3.2.7b Optimising conditions for RPAs.

Conditions such as concentration of RNA and amount of antisense probes, to be used in the RPA assays were established using liver RNA from CBA/Ca mice (Figure 3.12). The results in these optimisation experiments show that bands of the expected size (278 nucleotides) are detected when 20 μg of RNA and 200 000 counts per minute (cpm) of the labelled 3’ antisense probe are used. The intensity of this band increased linearly when 20 μg of RNA were incubated in the presence of 400 000 cpm of labelled probe. The intensity of this band did not change substantially when 40 μg of RNA was incubated with the same amount of probe suggesting that the probe was saturated beyond ~25 μg of RNA.
Figure 3.11. Schematic representation of constructs made to generate GPI-PLD antisense probes. The 5' probe (A) was made by cloning a 434-bp fragment (from nucleotide 52 to 486) into the *Pst* I/*Cla* I sites of the pBluescript SK' vector. The plasmid was digested with *Pvu* II to generate an antisense probe of 740 bp.

The probe for the middle part of the GPI-PLD was generated by cloning a 384-bp fragment (from nucleotides 1009 to 1393) into the *Srf* I site of the pCRScript SK' vector. After digestion with *Pvu* I and *Sac* I, an antisense probe of 468 bp was synthesised (B).

The RNA probe for the GPI-PLD 3' end was generated by cloning a 278 bp fragment (from nucleotide 2713 to 2994) into the *Bam* HI/*Sal* I sites of the pCRScriptSK' vector. A 565 bp antisense probe was synthesised after digestion with *Pvu* II (C).

All the anti sense probes were synthesised by using the T7 RNA polymerase. The 3' and 5' ends of the GPI-PLD fragments are indicated to shown the orientation within the pBluescript SK' and pCRScript SK' plasmids. Short segments of the pancreas and liver GPI-PLD sequences where differences were found are shown in red. The red arrow indicates the direction of synthesis from the T7 promoter.
This means that by using these assay conditions, 400 000 cpm of antisense probe and 20 µg of RNA, the labelled probe is present in a molar excess in the reaction, and can under these conditions give an accurate quantification of the amount of GPI-PLD mRNA present in the total RNA sample. For the 5' anti sense probe, no increase in the concentration of protected fragment was detected when 40 µg of RNA were incubated with 400 000 cpm if compared with 20 µg RNA and the same amount of antisense probe (Figure 3.12). As the incubation of 40 µg of RNA in the presence of 400 000 cpm did not increase the protected fragment either, this means that saturation was reached with ~250 000 cpm and 20 µg of RNA. For the actin probe, the assay conditions where a band of the expected size is detected and the probe is in molar excess were with 20 µg of RNA in the presence of 50 000 cpm of the antisense probe (Figure 3.12). Based on this, these conditions for each antisense probe were used in further experiments.

The specificity of the assay is demonstrated in the reactions where the 3', 5' or actin antisense probes were incubated with yeast RNA only. The absence of bands in the lanes corresponding to these reactions demonstrates that no unspecific binding occurs under the assay conditions used. These optimisation experiments also demonstrate the efficiency of the ribonuclease digestion, at least for the 3' and 5' antisense probes. In the case of the actin probe and for unknown reasons, the complete degradation of the probe was not achieved. In these and subsequent experiments, part of the undigested probe was always detected.
Figure 3.12. Establishment of the optimal conditions for detection of RNA protected fragments in Ribonuclease Protection Assays (RPA). Either 20 or 40 μg of total liver RNA from CBA/Ca mice were incubated with a 3’ or a 5’ antisense GPI-PLD labelled probes at an activity of 200 000 or 400 000 counts per minute (cpm). For the actin probe, 10 000 to 50 000 cpm were used. When using 20 μg of RNA, 20 μg of yeast RNA were added to have a final concentration of 40 μg of RNA per reaction. 726, 882 and 200 (ribonucleotides) indicate the molecular size of the undigested 3’, 5’ and actin probes, respectively. 278, 434 and 150 (ribonucleotides) indicate the size of the protected fragments obtained after digestion for the 3’, 5’ and the actin probes, respectively.
3.2.7c GPI-PLD expression in different mouse tissues.

Having established the optimal conditions for the RPA, the expression of GPI-PLD was analysed in different tissues from CBA/Ca mice. As shown in Figure 3.13, the 3' and 5' probes protect a 278 and a 434 ribonucleotide fragments, respectively. In addition to the 5' protected fragment band, a band of slightly lower molecular weight was detected. Incubation with individual probes showed that this band is related to the 3' probe and probably is the result of incomplete digestion. The different intensity of the bands corresponding to the 5' and 3' protected fragments could be reflecting a higher stability of the RNA/probe complexes formed with the 3' probe.

According to this analysis, GPI-PLD is present in the liver and brain RNA samples and in lower degree in antrum and duodenum, but not in RNA derived from other tissue samples. These results indicate, furthermore, that the GPI-PLD mRNA sequence expressed in mouse liver, brain, duodenum and antrum is identical to the 5' and 3' antisense probes that were derived from the consensus sequence. These results were corroborated in another set of experiments using a different GPI-PLD antisense probe that contains the 6-bp insertion detected in the clone 19 GPI-PLD consensus sequence. The results of the analysis with the antisense probe for the middle part of the GPI-PLD sequence (Figure 3.14) confirm that liver and brain are the major tissues producing GPI-PLD. Faint bands were also detected with antrum RNA samples as observed above.
Figure 3.13. Ribonuclease protection assay for detection of GPI-PLD in different tissues from CBA/Ca mice. A) The expression of GPI-PLD in different tissues from CBA mice. A, antrum; B, brain, D, duodenum; H, heart, K, kidney; Li, liver, Lu, lung; Sg, salivary gland; Sm, skeletal muscle; Sp, spleen, T, thymus. B) Densitometry quantification of the GPI-PLD expression. The relative level of GPI-PLD expression determined with 3' probe (□) and the 5' probe (■) is shown as a ratio of the intensity of GPI-PLD and actin bands determined by PhosphorImaging. C) Schematic representation of the GPI-PLD gene and the position of the regions covered by the antisense probes. The antisense probes were designed based on the consensus sequence obtained in this work. The gray area in the probes correspond to pBluescript SK⁺ and pCRScript SK⁺ plasmid sequences.
Figure 3.14. GPI-PLD expression in tissues from CBA/Ca mice. A) Tissue specific expression of GPI-PLD detected by ribonuclease protection assay in different tissues from CBA/Ca mice. A, antrum; B, brain; D, duodenum; H, heart; K, kidney; Li, liver; Lu, lung; P, pancreas; Sg, salivary gland; Sm, skeletal muscle; Sp, spleen; T, thymus. B) Densitometry quantification of the GPI-PLD. The relative level of GPI-PLD expression is shown as a ratio of the intensity of GPI-PLD and actin bands. C) Schematic representation of the GPI-PLD gene and the position of the region covered by the antisense probe corresponding to the middle part of GPI-PLD. The antisense probe was designed based on the consensus sequence obtained in this work. Up, undigested probes. Similar results were obtained in two independent experiments.
In this experiment a sample of RNA derived from pancreatic tissue was included. Interestingly, no bands were detected in this sample, suggesting that the expression level was much lower than that observed in liver and brain. Another important observation from these experiments is that no predicted fragments derived from a pancreas sequence were detected. This indicates that the pancreas sequence was not present in any of the different tissues analysed.

3.2.8 Sequence analysis of GPI-PLD fragments obtained from different tissues by RT-PCR.

The analysis of GPI-PLD RNA expression in different tissues by the RPA assays did not give any evidence that the sequence differences are due to a tissue-specific expression pattern. With this in mind, the most likely explanation is that there are sequencing errors in the published GPI-PLD pancreas-derived sequence and that the consensus sequence obtained in this work is correct. More evidence for this hypothesis comes from RT-PCR and sequencing experiments. Using RT-PCR on total RNA from liver, brain and a pancreatic cell line, two fragments of the GPI-PLD were amplified. The first fragment (1028 bp) was obtained using a pair of primers (Sac55 and Sal 33) that amplified a region containing the 12-nucleotide insertion detected in the liver GPI-PLD consensus sequence. The other fragment (603 bp) was obtained with a set of primers (Sal53 and V5') that amplified a region containing the 3-nucleotide deletion and the 6-nucleotide insertion detected in the liver GPI-PLD consensus sequence (Figure 3.15). In these experiments, no controls for contamination of RNA samples with genomic DNA were included because the regions between the primers used are separated by several large introns (Schofield and Rademacher, 2000) and would not result in the generation of PCR products.

The amplified fragments were purified from agarose gels and sequenced. The results confirmed the previous determined sequence (Figure 3.3). In addition, the sequences...
of the PCR products obtained from the pancreatic cell line were also completely
homologous with the GPI-PLD clone 19 consensus sequence. This would strongly
support the hypothesis that only one GPI-PLD gene exists with the sequence as
determined for clone 19 (Figures 3.3 and 3.4). The pancreas-derived published
sequence must therefore contain sequence errors.

Figure 3.15. Detection of GPI-PLD by RT-PCR. 2 \( \mu \)g of total RNA were used in the reverse
transcriptase (RT) reaction. One tenth of the reaction volume, containing the cDNA, was used
for the PCR. A) Lanes 1 and 14, 1kb DNA ladder; lanes 2 and 8 brain from CBA/Ca mice; lanes
3 and 9, liver from CBA/Ca mice; lanes 4 and 10 pancreatic cell line NIT-1; lanes 5 and 11 brain
from NOD mice; lanes 5 and 12, liver from NOD mice; lanes 7 and 13, negative controls, where
no RNA was included in the RT reaction. B) The RNA for the RT reaction was obtained from
macrophage cell lines P388 (lanes 2 and 5) or RAW264.7 (lanes 3 and 6). Lanes 1 and 4,
negative controls, where no RNA was included in the RT reaction.
3.2.9 GPI-PLD expression in non-diabetic mouse strains.

In an attempt to discover if genetic factors affect the expression of GPI-PLD, RPAs were used to analyse the level of liver GPI-PLD mRNA in different inbred mouse strains. By using a probe for the 3' end, it should be possible to look for allelic variations, at least within the sequences included in the antisense probes, and the expression level could indicate genetic regulation of GPI-PLD expression.

A)

![Image showing RPA analysis of GPI-PLD expression in different mouse strains.](image)

B)

![Image showing densitometry quantification of GPI-PLD mRNA levels.](image)

**Figure 3.16.** Expression of GPI-PLD in liver of different mouse strains. A) Total RNA was extracted from liver samples obtained from 8-10-week old male mice. A GPI-PLD antisense RNA probe for the 3' end was used as indicated in Figure 3.13. BL10, (C56BL/10). B) Densitometry quantification of GPI-PLD mRNA levels expression. The relative level of GPI-PLD expression is shown as a ratio of the intensity of GPI-PLD and actin bands. Similar results were obtained in two independent experiments.
Figure 3.16 shows that normal mouse strains such as CBA/Ca and C56BL/10 express a relatively high level of GPI-PLD in the liver. This is in contrast to NZW mice which showed the lowest levels of GPI-PLD expression, and BALB/c, DBA/1 or DBA2 strains, which have a lower steady state level of GPI-PLD mRNA in the liver. The fact that no GPI-PLD sequence differences were observed among these mouse strains, at least in the region covered by the antisense probe, suggests that no allelic variants of GPI-PLD exist in these mouse strains. These results also suggest that genetic factors may be important in regulating GPI-PLD expression.

3.2.10 GPI-PLD expression in diabetic mice

Further evidence of the importance of the genetic background in the control of the expression of GPI-PLD was obtained when the GPI-PLD mRNA level in the liver of nonobese diabetic mice (NOD) was analysed.

NOD mice spontaneously develop insulin-dependent diabetes and are a model of type I diabetes mellitus. In this experiment two groups of NOD mice were analysed, those which had not developed diabetes (4-17 weeks), and those that after 20 weeks had become diabetic, but had not been diabetic for long time. Results obtained using a probe for the 3' end (Figure 3.17A) show that GPI-PLD is expressed at a high level in non-diabetic young mice (4 weeks), when compared to older non diabetic animals (8-17 weeks). This result may indicate that hormonal factors may be important in the regulation of GPI-PLD mRNA levels. High levels of GPI-PLD mRNA levels were also detected in diabetic mice relatively to non-diabetic mice (1.5-2 fold increase). When compared to actin RNA levels, this high level of GPI-PLD expression is seen especially at 28 weeks (Figure 3.17B). These results may suggest that down-modulation of insulin or up-regulation of glucose, typical characteristics of the diabetic state, increase the level of GPI-PLD mRNA in vivo.
To further examine this, the expression of GPI-PLD in the liver of diabetic animals was analysed. First, streptozotocin (STZ)-treated BALB/c mice which constitute a model of chemically-induced diabetes were analysed. STZ is a glucosamine-nitrosourea compound (Lewis and Barbiers, 1960), which as an unstable molecule accumulates in pancreatic β cells and induces the generation of free radicals, such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO) which result in DNA fragmentation and a cytotoxic effect on pancreatic cells (Nukatsuka et al., 1988). STZ has been used as an agent to induce experimental animal diabetes with clinical characteristics similar to those observed in the autoimmune form (type 1) of the disease (Haluzik and Nedvidkova, 2000).

In the second model, ob/ob (deficient for leptin) and db/db (deficient for leptin receptor) mice were analysed. In these mice, the deficiency of leptin or its receptor results in severe obesity, hyperinsulinemia, and hyperglycemia in a similar way to that observed in type 2 diabetes (Seufert et al., 1999). STZ-induced diabetic BALB/c mice showed an increase in the level of expression of GPI-PLD RNA relative to untreated BALB/c mice (Figure 3.18A and B). The opposite effect however, was observed when liver samples from ob/ob and db/db mice were analysed. (Figure 3.18 C and D). When compared to normal CBA/Ca mice, ob/ob and db/db mice expressed lower levels of GPI-PLD RNA. These results suggest that insulin is responsible for GPI-PLD expression in these animal models of diabetes. In diabetic animals with low levels of insulin, GPI-PLD RNA is present at high levels in liver, whilst the opposite affect, low levels of GPI-PLD RNA in liver, are seen in animal model with high levels of insulin.
Figure 3.17. A) Ribonuclease Protection Assay (RPA) for detection of GPI-PLD expression in liver from NOD mice at different ages (weeks). up, undigested probes. NOD mice from 4-17-week old had not developed diabetes when tissues were dissected. From 20 weeks of age, NOD mice had developed diabetes. B) Radioactive intensity for GPI-PLD and actin bands was quantified in a phosphoimager. The GPI-PLD/actin radioactivity ratio is expressed. Similar results were obtained in two independent experiments.
Figure 3.18. Analysis of GPI-PLD mRNA expression in the liver of diabetic mice strains. A) Ribonuclease protection assay for detection of liver GPI-PLD in streptozotocin (STZ)-induced diabetic BALB/c mice. STZ was dissolved in sterile citrate buffer and injected intraperitoneally into BALB/c (40 mg/kg, ~20 µl) within 5 min of preparation. STZ or citrate buffer alone (Control) was administered for 5 consecutive days. B) Ribonuclease protection assay for detection of liver GPI-PLD in non-dependent insulin diabetic ob/ob and db/db mice strains. Total RNA was extracted from liver samples obtained from 8-10-week old male mice. C) and D) Densitometry quantification of GPI-PLD mRNA levels. The relative level of GPI-PLD expression is shown as a ratio of the intensity of GPI-PLD and actin bands. GPI-PLD antisense RNA probes for the 5' and 3' ends were used as indicated in Figure 3.13.
3.3 DISCUSSION

3.3.1 Mouse liver GPI-PLD DNA sequence.

Sequence analysis of a full-length transcript cDNA clone (clone 19) isolated from a mouse liver cDNA library and with homology to GPI-PLD, showed sequence differences when compared to a mouse pancreas-derived GPI-PLD (LeBoeuf et al., 1998). The sequence differences resulted in several single nucleotide changes and the insertion of 12- and 6-nucleotide fragments, and the deletion of a 3-nucleotide fragment in the liver-derived sequence (Figure 3.3).

Reports on the expression of at least two GPI-PLD RNA transcripts in bovine liver (Stadelmann et al., 1993) and the observation of DNA sequence differences in human liver and pancreas-derived GPI-PLD clones (Tsang et al., 1992), led to the suggestion that either GPI-PLD could be encoded by multiple genes or be expressed as alternatively-spliced forms of a single gene in tissue-specific manner. It was decided therefore to design a series of experiments in order to determine which one of these suggestions would explain the sequence differences observed between the pancreas-derived mouse GPI-PLD sequence previously reported (LeBoeuf et al., 1998), and the one obtained in this thesis.

Digestion with Bam HI restriction enzyme and Southern blot analysis of genomic liver DNA from CBA/Ca mice showed the presence of many faint but one dominant hybridising band (Figure 3.8). In a recent paper, the complete gene structure of the human GPI-PLD has been described. The gene encompasses 24 short exons (27 to 187 bp) and a 3224-bp final exon (Schofield and Rademacher, 2000). In addition to describing the human GPI-PLD gene structure, in that study conclusive evidence of only one gene coding for GPI-PLD was obtained in PCR experiments and Southern blot analysis of human genomic DNA and a P1-derived artificial chromosome (PAC) containing human GPI-PLD exons 15-25 (Schofield and Rademacher, 2000).
As most of homologous genes are conserved among species, the mouse GPI-PLD gene might very well have the same size and contain an equal number of small exons separated by relatively large introns. Such a structure would be consistent with the Southern blot result shown in Figure 3.8.

If as in humans, only one gene codes for mouse GPI-PLD, the sequence differences found between pancreas- and liver-derived GPI-PLD forms could be the result of the synthesis of different transcripts. The existence of two alternately spliced forms of the phosphatidylcholine (PC)-specific PLD has been demonstrated (Hammond et al., 1997). The possibility that such a process occurs for mouse GPI-PLD was evaluated by Northern blot and RPAs.

Northern blot results showed that among eleven different tissues tested only one transcript of ~4 kb in liver, and one of a slightly higher molecular weight in brain, were detected. These results do not correlate with those reported previously where three transcripts of 3.9, 5.4 and 8 kb, respectively, were detected in liver and other tissues (LeBoeuf et al., 1998). This discrepancy can be explained by the type of RNA and the probes used for the experiments: In this work total RNA was used, whilst Le Boeuf et al., employed poly (A)^+ RNA, where the specific mRNAs are enriched. When using poly (A)^+ RNA it is possible to load more RNA in the gel resulting in higher sensitivity.

It is highly likely that the ~4 kb transcript detected in this thesis is identical to the 3.9 kb transcript described by Le Boeuf et al. Supporting this, the detection of only one GPI-PLD transcript produced mainly in liver and brain has been also reported in humans (Tujjoka et al., 1998; Schofield and Rademacher, 2000). The slight difference in molecular size between the liver- and brain-derived GPI-PLD transcripts observed in the Northern blot analysis has also been reported (Schofield et al., 2002). This different size might indicate the generation of tissue-specific transcripts. These differences must affect regions of the mRNA not included in the antisense probes (such as the 3' non-
translated regions or the 5' end which could include tissue specific promoters) otherwise these differences could had been detected in the RPAs.

The 5.4 and 8 kb transcripts detected by Le Boeuf *et al.*, but not detected in this thesis may either represent GPI-PLD transcripts not completely spliced, or cross-hybridisation with other mRNA molecules. It could be possible that a the complete GPI-PLD sequence used as a probe by Le Boeuf *et al.* shows limited cross-reactivity with other molecules within the 5' and 3' non-translated sequences. Supporting this idea, analysis of clones containing genomic GPI-PLD sequences has shown that it contains part of the gene encoding for NAD⁺-dependent succinic semi-aldehyde dehydrogenase (clone AL031230, NCBI Databank). By using a shorter probe, like the 972-bp probe used in this thesis, cross-reaction with other molecules could be avoided.

The detection of protected fragments of the expected size in the RPAs further support that the sequence differences between pancreas and liver GPI-PLD are not due to the generation of tissue-specific GPI-PLD forms. The most probable explanation is that the sequence differences are due to sequencing errors in the pancreas-derived GPI-PLD. This was corroborated by RT-PCR amplification and sequencing of GPI-PLD fragments from liver and pancreas containing the sequence differences (Figure 3.15).

When GPI-PLD 5' and 3' antisense probes were used together in the RPAs, a strong band corresponding to the 3' protected fragment was detected. If only one GPI-PLD transcript is expressed, as observed in the Northern blot, then bands for the 5' and 3' ends with the same intensity would be expected. At first, it was believed that the strong intensity of the 3' probe was due to the detection of additional transcripts. If this were the case however, the 3' probe used in this assay would not allow their detection. As the percentage of [α-³²P]-CTP incorporated in each probe is almost the same, ~25% of the total sequence, the strong intensity of the band corresponding to the 3' end is most
probably due to artefacts in the RPA. It is also possible that the liver RNA/5’ antisense probe complexes are more susceptible to the action of ribonucleases.

Taken together the results from the Southern and Northern blot, RPA assays, comparison of EST clones, and RT-PCR it can be concluded that:

- Mouse GPI-PLD is encoded by only one gene, which is processed into a single transcript, possibly in a tissue-specific manner.
- The sequence differences between the pancreas and liver-derived GPI-PLD sequences are the result of sequencing errors in the pancreas sequence.

3.3.2 Mouse liver GPI-PLD protein sequence.

The DNA sequence differences between pancreas- and liver-derived GPI-PLD resulted in some amino acid changes, but despite this the reading frame was not altered (Figure 3.4). As already mentioned with the DNA sequences, the fact that the amino acid differences found in the mouse liver-derived sequence are shared with the human and bovine GPI-PLD sequences indicates that the sequence obtained in this work is correct.

The DNA sequence predicted for an 840-amino acid polypeptide that yields an 817-amino acid mature protein after the cleavage of the 23-amino acid signal sequence. Search in databases for motifs within the sequence, revealed that the mouse liver-derived GPI-PLD sequence obtained in this work, shows four regions of internal homology that have similarity with the metal-ion binding site (Figure 3.6) described in the alpha subunits of integrins (Scallon et al., 1991). Analysis of metal ions content and results from studies of inhibition of the enzymatic activity by cation chelators, have suggested that these sites are functional in the GPI-PLD and that they are not related with the enzyme activity, but with the maintenance of the tridimensional structure (Li et al., 1994; Li et al., 1999).
Potential sites of phosphorylation were also found (Table 3.1). The establishment of actual modification sites by protein kinases will be very helpful to understand the mechanisms that regulate the stimulation/inhibition of GPI-PLD activity. In fact, it has been demonstrated that GPI-PLD can be phosphorylated \textit{in vitro} by cAMP-dependent protein kinase and by tyrosine kinase (Civenni \textit{et al.}, 1999).

Further analysis of the protein sequence revealed the presence of the motif $\Psi X \cdots X Y$, where $\Psi$ represents an aromatic residue and $X$ any other amino acid (Figure 3.4), that is shared by many different proteins regulated by caveolin (Couet \textit{et al.}, 1997a), the major structural component of specialised membrane domains termed caveolae (Schlegel and Lisanti, 2001). The presence of this caveolin-binding motif in GPI-PLD would suggest a potential interaction between these two molecules. Supporting this idea, recent work has demonstrated that PLD2, a splicing isoform of PLD, is specifically localised in caveolae (Czamy \textit{et al.}, 1999; Kim \textit{et al.}, 1999) which is also enriched in GPI-anchored proteins (the potential substrates of GPI-PLD) (Brown and Rose, 1992; Ying \textit{et al.}, 1992). However, GPI-PLD does not contain any motifs for lipid modification (palmitoylation nor myristoylation), and being GPI-PLD a secreted protein, it would not interact with the cytoplasmic enzymes (Farazi, \textit{et al.}, 2001) in charge of these lipid modifications. Both of these lipid-modifications are an important feature of caveolin-interacting proteins (Okamoto \textit{et al.}, 1998), suggesting that GPI-PLD may not interact with caveolin. In addition, GPI-PLD and caveolin are situated in opposite of the membrane, which would make difficult an interaction between these two molecules.

\textbf{3.3.3 GPI-PLD expression.}

Liver and brain are the two main tissues where GPI-PLD expression was detected. GPI-PLD mRNA was also detected in antrum, lung and duodenum although at much lower levels (Figures 3.9, 3.13 and 3.14). Despite some discrepancy related with the number of bands detected (LeBoeuf \textit{et al.}, 1998) when analysing GPI-PLD mRNA
expression by Northern blotting, the results in this work agree with the fact that liver
seems to be the major source of both mouse and human GPI-PLD. This was confirmed
when an EST database from the NCBI was analysed. Over 100 different EST clones
with homology to GPI-PLD have been deposited in this database. About 30% of these
clones are derived from liver samples (Table 3.2). However, after analysing the tissue
sources from where the rest of the EST clones were obtained, it is clear that GPI-PLD
is widely expressed in many different tissues, albeit at such low levels that its detection
was not possible with the methods employed here. Indeed, RT-PCR analysis (see
Figure 3.15A and B) using pancreatic β-cells (NIT-1 cell line) and macrophages (P388
and RAW264.7 cell lines) mRNA as templates allowed the identification of the GPI-PLD
transcripts in these cells. The different relative amounts of mRNA expressed in brain
and liver suggest that tissue-specific mechanisms (different promoters or binding
proteins) regulating the expression of GPI-PLD might exist.

The final experiments described in this chapter were undertaken to try to find out
whether the genetic background of different mouse strains can affect the GPI-PLD
RNA production in the liver. The results showed that normal mouse strains such as
C57BL/10 and CBA/Ca express relatively high levels of GPI-PLD when compared to
the other normal strain analysed, BALB/c. This level of expression was even higher if
compared to NZW (which showed the lowest levels of GPI-PLD expression), DBA/1
and DBA/2 mouse strains (Figure 3.16).

One interesting possibility is that insulin and/or glucose concentrations are responsible
of such variations in the GPI-PLD expression levels among different mouse strains.
NZW mice when crossed with NZB mice generate F1(NZBxNZW) mice which are
highly susceptible to develop autoimmune diseases such as lupus erythematosus. It
has been recently shown that New Zealand Obese (NZO) mice, a strain close related
to NZW mice, develop obesity, glucose intolerance, insulin resistance and have a high
tendency to develop type-2 diabetes (Haskell et al., 2002). Variations in the levels of
glucose and insulin have also been detected in DBA/1 mice, which develop type 2 collagen-induced arthritis (Murray et al., 1993). As can be seen, differences in the GPI-PLD expression levels exist among diverse mouse strains and most probably these differences are associated with the genetic background of each mouse strain.

Additional evidence that insulin and/or glucose levels may be important in determining GPI-PLD expression was obtained when male nonobese diabetic (NOD) mice (a model of type 1 diabetes) were analysed (Figure 3.17). The results show that GPI-PLD mRNA in liver is increased as mice become diabetic, suggesting that metabolic changes associated with the onset of diabetes are important in the regulation of GPI-PLD expression. The analysis of liver GPI-PLD expression in another model of type 1 diabetes gave similar results. STZ-induced diabetic mice showed a relative increase in the level of GPI-PLD expression when compared to control untreated animals (Figure 3.18A). This observation is in agreement with a recent work where similar results were obtained (Deeg et al., 2001). The onset of the disease in these two models involves the immune system-mediated destruction of pancreas, resulting in insulin-dependent diabetes mellitus. Based on this, it has been proposed that insulin and/or glucose levels might play a role in the regulation of GPI-PLD production in vivo, at least in these models of autoimmune disease (Deeg et al., 2001).

Further evidence of a possible regulatory role of insulin and/or glucose on the expression of GPI-PLD was obtained from the analysis of liver GPI-PLD mRNA expression in two animal models of type 2 diabetes. This metabolic disease, also known as non insulin-dependent diabetes, is characterised by insulin resistance and over production of this hormone. The slight decrease in liver GPI-PLD mRNA observed both in ob/ob and db/db mice is in agreement with results obtained by Bowen et al., when analysing GPI-PLD expression in pancreatic islets and liver in ob/ob mice (Bowen et al., 2001). An inhibitory role of insulin on liver GPI-PLD expression is further supported by the observation that administration of insulin to STZ-induced diabetic rats
decreased the high levels of serum GPI-PLD and mRNA in liver of these animals (Schofield et al., 2002). One interesting observation in the study of ob/ob mice by Bowen et al., is the in vivo stimulatory effect of insulin on the expression of GPI-PLD by pancreatic cells (Bowen et al., 2001). These results and the previous description of the in vitro stimulatory effect of insulin on GPI-PLD mRNA expression in β pancreatic cells (Deeg and Verchere, 1997) suggest that control of GPI-PLD expression is tissue-specific, at least in these diabetes animal models.

Another possible link between GPI-PLD expression and insulin/glucose levels comes from studies on the variations of IPG A-type and IPG P-type levels detected in diabetic patients (Aspin et al., 1993; Kunjara et al., 1999). As already described, IPGs with a second messenger activity in the insulin signal transduction pathways, are the products of the enzymatic activity of GPI-PLD on free GPls (Mato et al., 1987; Pak and Larner, 1992). Although the relationships among IPGs, insulin/glucose and GPI-PLD are not clear, the increment in the levels of IPG A-type in type 1 diabetes could be a response to the increased levels of GPI-PLD, which in turn seem to be related to the decreased levels of insulin described in this disease. On the other hand, in type 2 diabetes, low levels of IPGs could be due to the decreased level of GPI-PLD. In fact, in mice models of this type of diabetes, the administration of plasmodium-derived IPGs reversed the disease by normalising the levels of glucose (Elased et al., 2001).

Diabetes, however, is a complex disease where multiple changes in the metabolism occur and it can not be ruled out that some other factors affect GPI-PLD expression. All this information points to the fact that the mechanisms regulating the expression of GPI-PLD are complex and diverse. The recent publication of the GPI-PLD gene structure and complete sequence will help to design experiments looking for and testing promoters or repressors of GPI-PLD expression.
CHAPTER 4

GENERATION OF MOLECULAR TOOLS TO STUDY

GPI-PLD

4.1 INTRODUCTION.

GPI-PLD is a highly specific enzyme presumed to be responsible for the cleavage of the free-GPI moieties present in the cell membrane (Jones et al., 1997) or the GPI-anchors used for a great variety of membrane proteins (Davitz et al., 1987; Deeg et al., 1995). So far, GPI-PLD has been cloned from bovine, human, and mouse sources. In the three species, GPI-PLD cDNA sequences predict for proteins of ~90 kDa with multiple potential sites of N-glycosylation, resulting in the detection of mature proteins of 100-115 kDa (Scallon et al., 1991; Tsang et al., 1992; LeBoeuf et al., 1998).

In Chapter 3 of this thesis and in other studies it has been demonstrated that liver is the main site of production of GPI-PLD (LeBoeuf et al., 1998; Schofield and Rademacher, 2000). It has been also shown, however, that other tissues such as brain, lung, antrum and macrophage and pancreatic β-cell lines (Chapter 3, Figure 3.9 and Table 3.2) are able to synthesise this enzyme (Hoener et al., 1990; Metz et al., 1991; Stadelmann et al., 1993; Rhode et al., 1999). The contribution of these tissues to the circulating GPI-PLD is not known because variations in the levels of circulating GPI-PLD in patients with liver diseases indicate that liver is the major source of serum GPI-PLD (Raymond et al., 1994; Maguire and Gossner, 1995).

GPI-PLD is highly abundant in serum, where it is found forming small particles/complexes in association with high-density lipoproteins. Chromatography and electrophoresis analysis of these complexes revealed that GPI-PLD associates with
apolipoproteins apoA-I and apoA-IV (Hoener et al., 1993; Deeg et al., 2001a). It has been also demonstrated that apoA-I stimulates in vitro the GPI-PLD-mediated cleavage of purified acetylcholinesterase, a GPI-anchored protein (Hoener et al., 1993). This effect, however, has not been shown for GPI-anchored proteins on the surface of intact membranes, and then the biological significance of this association remains to be established.

It has been speculated that apoA-I directs GPI-PLD to specific compartments where it encounters and cleaves the GPI-anchored substrates in a membrane environment different to that in the cell surface (Deeg et al., 2001a). Studies on the possible GPI-PLD intracellular site of action have provided evidence of an uptake mechanism in which GPI-PLD is taken up from media by neuroblastoma cells. In this model, GPI-PLD is internalised in a concentration- and time-dependent way, becoming active on GPI-anchored proteins only after its proteolytic processing in a not yet identified intracellular compartment (Hari et al., 1997). This model is supported by the observation that in vitro treatment of GPI-PLD with trypsin generates three different fragments without affecting the enzymatic activity (Hoener et al., 1994; Li et al., 1994; Heller et al., 1994). On the other hand, co-transfection studies where GPI-PLD and GPI-anchored proteins are expressed simultaneously suggest that GPI-PLD action occurs within compartments of the secretory pathway as proteins are being transported to the exterior (Scallon et al., 1991; Bernasconi et al., 1996; Kung et al., 1997).

From all these studies and as GPI-PLD is abundant in bovine serum, which is normally used when culturing cells, it is not clear whether the reported release of GPI-anchored proteins is due to endogenous GPI-PLD or extracellular GPI-PLD taken up by the cells (Xie and Low, 1994).
The identification of GPI-PLD-producing cells and the precise determination of the intracellular site(s) where this enzyme is localised are very important to understand the mechanisms that regulate the GPI-PLD activity in vivo.

Considering this, the aims of the experiments presented in this chapter are: to design plasmid constructs encoding for GPI-PLD fusion proteins expressing different tags recognised by specific antibodies and to demonstrate their functional activity. The design and generation of suitable GPI-PLD recombinant fusion proteins will allow an adequate analysis of the expression and activity of GPI-PLD in transfected cells.
CHAPTER 4. GENERATION OF MOLECULAR TOOLS TO STUDY GPI-PLD

4.2 RESULTS.

4.2.1 Generation of GPI-PLD plasmid constructs.

One common technical problem to analyse the expression of cloned cDNAs is the lack of specific antibodies to the protein produced. To overcome this problem, fusion proteins where a known peptide sequence or epitope (tag) is fused to the expression product have been described (Witzgall et al., 1994).

In the particular case of mouse GPI-PLD, its detection and analysis of intracellular localisation have relied on the use of antibodies against the human or bovine form (Xie and Low, 1994; O'Brien et al., 1999; Deeg et al., 2001b). To make the analysis of mouse GPI-PLD more specific, in this work several GPI-PLD plasmid constructs containing different sequence tags have been made. The production of GPI-PLD fusion proteins incorporating specific tags and the use of commercially available anti-tag specific antibodies would allow an adequate analysis of the expression and intracellular localisation.

The plasmid construct pcDNA3/GPI-PLD was made to analyse the GPI-PLD activity on transfected cells. The incorporation of GPI-PLD into this vector allows a high and efficient expression in mammalian cells. In addition, the pcDNA3/GPI-PLD/Myc-His A, pGPI-PLD/EGFPN3 and pCMV83XFlag/GPI-PLD plasmid constructs were designed to synthesise GPI-PLD fusion proteins as molecular tools suitable to analyse GPI-PLD expression in transfected cells. The incorporation of green fluorescent protein (GFP), Myc or 3XFlag tags allows the microscopic analysis either by direct (GFP) or indirect (Myc and 3XFlag) fluorescence. The use of specific antibodies for these three tags also facilitated the analysis of GPI-PLD by biochemical approaches.
Figure 4.1. Schematic representation of GPI-PLD-plasmid constructs made and used in this study. The main structural features of each plasmid are indicated and the sequence corresponding to the translated GPI-PLD is shown in light blue, whilst the 5' and 3' end untranslated sequences are shown in dark blue. The restriction enzyme sites where GPI-PLD was cloned are indicated for each plasmid. A) pSPORT1/GPI-PLD contains the entire GPI-PLD sequence obtained from a mouse liver library. B) pPROEXHTc/GPI-PLD is the construct containing the entire GPI-PLD coding sequence without the stop codon. C) The pSPORT1/GPI-PLD Δ ss construct contains the GPI-PLD sequence lacking the signal sequence.
**Cont. Figure 4.1** D) pcDNA3/GPI-PLD, the EcoRI/NotI fragment of GPI-PLD clone 19, corresponding to the entire GPI-PLD sequence was inserted into EcoRI/NotI sites of pcDNA3 plasmid. E) pcDNA3/GPI-PLD/Myc-His A construct was generated by inserting the EcoRI/XhoI fragment from B) into the EcoRI/XhoI sites of the pcDNA3-Myc-His A plasmid. F) The EcoRI/XhoI fragment from B) was cloned into the EcoRI/SalI sites of the pEGFP-N3 to generate the construct pGPI-PLD/EGFP-N3. G) The p3XFlag-CMV-8/GPI-PLD was generated by inserting a GPI-PLD fragment lacking the signal sequence into the SalI/SmaI sites of the p3XFlag-CMV-8 plasmid.
The pcDNA3/GPI-PLD construct was generated by digesting the GPI-PLD clone 19 (from the pSPORT1 plasmid, Figure 4.1A) with Eco RI and Not I restriction enzymes. This fragment, corresponding to the entire GPI-PLD sequence, was cloned into the Eco RI/Not I sites of the pcDNA3 plasmid (Figure 4.1D).

To make the pcDNA3/GPI-PLD/Myc-His A construct, it was necessary to generate a GPI-PLD fragment lacking the stop codon at the 3' end. For this, a 681-bp fragment corresponding to the GPI-PLD 3' end (nucleotides 1889-2569) was amplified by PCR using the II5' and II3' set of primers (see Figure 4.1). The PCR-amplified fragment incorporating a Bam HI and a Xho I restriction sites in the 5' and 3' ends, respectively, was inserted into the Bam HI/Xho I restriction sites of the pPROEXHTc plasmid.

The resulting plasmid was then digested with Eco RI and Sac I enzymes and ligated with a GPI-PLD Eco RI/Sac I fragment (derived from digestion of pSPORT1/GPI-PLD clone 19 plasmid) to generate the pPROEXHTc/GPI-PLD plasmid (Figure 4.1B). This plasmid contained the entire GPI-PLD coding region with a Xho I site at the 3' end before the stop codon. This new plasmid was digested with Eco RI and Xho I to obtain the GPI-PLD coding region which was cloned into the Eco RI/Xho I sites of the pcDNA3/Myc-His A plasmid. Figure 4.2 shows the cloning strategy to make this construct.

After ligation and transformation, cloning in the correct reading frame was checked by sequencing. Once expressed in mammalian cells, the pcDNA3/GPI-PLD/Myc-His A plasmid (Figure 4.1E) would produce a Myc-tagged GPI-PLD fusion protein recognised by anti-Myc specific antibodies.
Figure 4.2. Flow diagram showing the strategy for the construction of the plasmid pcDNA3/GPI-PLD/Myc-His A.
In order to make the pGPI-PLD/EGFP-N3 (Figure 4.1F), the same GPI-PLD fragment lacking the stop codon was used. The Eco RI/Xho I GPI-PLD fragment from the pPROEXHTc/GPI-PLD was cloned into the Eco RI/Sal I sites of the pEGFP-N3 plasmid (Figure 4.3). Once expressed in mammalian cells this plasmid will produce a green fluorescent/GPI-PLD fusion protein.

**Figure 4.3.** Flow diagram showing the strategy for the construction of the plasmid pGPI-PLD/EGFP-N3
To generate the p3XFlag-CMV-8/GPI-PLD plasmid construct (Figure 4.1G), a GPI-PLD fragment lacking the signal sequence was obtained. For this, one GPI-PLD fragment corresponding to the 5' end and lacking the signal sequence was amplified by PCR using the sense primer p432 and the reverse primer p418. The 417 bp (from 113 to 530 bp) PCR-amplified GPI-PLD fragment (containing a SalI and a Clai restriction sites at the 5' and 3' ends, respectively) was digested with this two restriction enzymes and ligated with the SalI/Clai digested pSPORT1/GPI-PLD plasmid to generate the pSPORT1/GPI-PLD Δss plasmid (Figure 4.1c). This plasmid was digested with SalI and SnaBI restriction enzymes and the resulting GPI-PLD fragment lacking the signal sequence was inserted into the SalI and SmaI sites of the p3XFlag-CMV-8 plasmid, which contains a signal sequence (Figure 4.4).

After ligation and transformation, cloning in the correct reading frame was checked by sequencing. Once expressed in mammalian cells, the p3XFlag-CMV-8/GPI-PLD plasmid (Figure 4.1G) will produce a 3XFlag-tagged GPI-PLD fusion protein recognised by anti-Flag specific antibodies.

4.2.2 Analysis of the expression of plasmid constructs

Once large amounts of the plasmid constructs were obtained by midi-prep they were used to analyse the expression of the predicted proteins by transient transfection into different mammalian cell lines. The expression of the different tagged-GPI-PLD constructs was analysed by SDS-PAGE and western blotting using tag-specific antibodies.
Figure 4.4. Flow diagram showing the strategy for the construction of the plasmid p3XFlag-CMV-8/GPI-PLD.
Transfection experiments were done using COS-7, 293T and BHK cell lines. COS cells were derived from the African green monkey kidney cells CV-1 by transformation with an origin-defective SV-40 virus, which has integrated into COS cell chromosomal DNA (Gluzman, 1981). Therefore, COS cells produce wild-type T antigen and SV40 origin-containing plasmids replicate in these cells to a high copy number 48 h post-transfection. COS-7 is a subline that produces a higher plasmid copy number that its related subline COS-1. COS-7 cells are efficiently transfected by the DEAE-dextran/chloroquine method yielding efficiencies of 50 to 70% (Aruffo, 1997). Expressed proteins in COS cells are biologically active, but because COS cells do not express the α-(1,3)fucosyltransferase which is capable of transferring fucose to either sialyl or asialyl precursors, they may not modify the expressed protein in the same way as the cell that would normally produce it (Goelz et al., 1990).

293 is a human embryonic kidney cell line which is transformed by the adenovirus E1A gene product. 293T is a subline which also express SV40 large T antigen, allowing replication of plasmids containing the SV40 origin and early promoter region. This cell line is highly transfectable by the calcium phosphate method with an up to 50% efficiency (Pear et al., 1993).

BHK-21 cells were obtained from hamster kidney cells and because of their high transfection efficiency they have been used as host cells for transformation with expression vectors containing selectable and amplifiable DNAs (Macpherson and Stoker, 1962). One particular characteristic of BHK cells is that they lack endogenous caveolin-3 (Cav-3). As it has been demonstrated that caveolins can form homooligomers which could potentially influence the distribution of introduced caveolins (Song et al., 1997), the lack of Cav-3 in BHK cells will facilitate the analysis of its possible association with GPI-PLD.
Results from transfection experiments show that COS-7, BHK and 293T cells (Figure 4.5A-C) transiently transfected with the p3XFlag-CMV-8/GPI-PLD construct expressed a Flag-tagged GPI-PLD fusion protein (Flag/GPI-PLD) of the expected size (~110 kDa), whilst non-transfected cells showed no bands. Flag-GPI-PLD protein could be detected at low levels 24 h post-transfection. The expression level, however, increased at 48 h and remained constant up to at least 72 h post-transfection (Fig 4.5A).

The Western blot analysis showed also that in addition to the major Flag/GPI-PLD polypeptide of the expected size, shorter forms of the GPI-PLD fusion proteins were also present. In COS-7, the relative molar ratio of the different Flag-immunoreactive bands was the same at 24, 48 and 72 h after transfection (Figure 4.5A). The size and the presence of immuno reactive bands is cell-specific because 293T and BHK showed a different pattern of bands with the 97 kDa protein as the most dominant band. This would suggest that the additional bands seen in transfected COS-7 cells most likely are the proteolytic fragments of the 97 kDa primary translated product (Figure 4.5B and C).

COS-7 cells were also transfected with the pGPI-PLD/EGFP-N3 plasmid and a GPI-PLD/GFP fusion protein of the expected size (~140 kDa) was detected 48 h after transfection (Figure 4.5D). As seen in cells transfected with the p3XFlag-CMV-8/GPI-PLD construct, additional bands of lower molecular size were also detected.
Figure 4.5. Western blot analysis of fusion proteins derived from p3XFlag-CMV-8/GPI-PLD and pGPI-PLD/EGFP-N3 plasmids. COS-7 (A and D), 293T (B), or BHK (C) cells (2.5X10^5) were transiently transfected in 6 well plates. After transfection, cells were collected at the indicated times. Cells were lysed in 1x SDS loading buffer. Samples were analysed in 12.5% polyacrylamide-SDS gels and blotted onto nitrocellulose membranes. Bands were detected with an ECL kit using specific antibodies for GFP and Flag tags. NT, non-transfected cells.
Cell extracts from cells transfected with the pCDNA3/GPI-PLD/Myc-His A construct were also collected 24 and 48 h after transfection and analysed by SDS gel electrophoresis and Western blot. No bands were detected (data not shown) irrespective of the mammalian cell lines used. DNA sequencing has confirmed that the Myc-tag is in frame, so the lack of detection may be caused by selective proteolysis that removes the C-end epitope tag. Indeed, very short GFP fragments are seen in Figure 4.5D, suggesting the presence of a dominant C-end proteolytic sequence.

As it is shown in Figure 4.6, the inability to detect the Myc-tagged GPI-PLD is not related with the anti-Myc antibody or the detection reagents and conditions used because a control Myc-tagged protein, pV22, is detected on the cell extracts of transfected 293T cells. It is possible that internal cleavage of GPI-PLD at the carboxyl end, where the Myc tag is located, make it impossible to detect the tagged protein if it is being produced by the transfected cells.

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**Figure 4.6.** Expression of Myc-tagged GPI-PLD proteins in transient transfected 293T cells. 293T cells were transfected either with pCDNA3-GPI-PLD/Myc-His A or pV22 Myc/His plasmid constructs. 48h after transfection cells and supernatants were collected and analysed in a 10% poly-acrylamide gel by SDS-PAGE. After electrophoresis, samples were blotted onto a nitrocellulose membrane, which was probed with a monoclonal antibody against Myc tag. Bands were detected with an ECL kit. CE, cell extract; SN, supernatant.
It could also be possible that once expressed, the GPI-PLD/Myc fusion protein adopts certain conformation that makes the Myc-tag no longer available to recognition by the monoclonal antibody anti-Myc used. Alternatively, extra C-terminal residues prevent proper folding of GPI-PLD, which therefore becomes targeted for proteolytic degradation.

4.2.3. GPI-PLD fusion proteins are released into the medium.

Previous studies have demonstrated that GPI-PLD is secreted into the medium by cultured cells (Scallon et al., 1991; Xie and Low, 1994; Kung et al., 1997). In order to determine if the GPI-PLD fusion proteins encoded by the plasmid constructs designed in this work could be secreted into the medium, cell extracts and supernatants from COS-7 cells transfected with the different GPI-PLD plasmid constructs were collected 48 h after transfection and analysed by SDS-PAGE and Western blot. The samples were transferred to a nitrocellulose membrane, which was re-probed with the different tag-specific antibodies. As previously indicated, COS-7 cells transfected with pGPI-PLD/EGFP-N3 (Figure 4.7A) or p3XFlag-CMV-8/GPI-PLD (Figure 4.7B), expressed proteins of the expected molecular weight (140 and 110 kDa, respectively). It is possible, however, that because of different glycosylation the secreted proteins showed a higher molecular weight compared to the cell-associated fusion proteins. Although a fraction of the expressed GPI-PLD/GFP and Flag/GPI-PLD was secreted into the medium, a significant fraction of the proteins remained cell-associated irrespectively of the epitope tag used or whether it is attached in the N- or C-end of the polypeptide chain. No products of lower molecular size to that predicted were detected in the supernatants.
Figure 4.7. Expression of tagged-GPI-PLD proteins in transient transfected COS-7 cells. COS-7 cells were transfected either with pCDNA3-GPI-PLD/Myc-His A, pGPI-PLD/EGFP-N3, p3XFlag-CMV-8/GPI-PLD or pEGFP-N3 plasmids. 48h after transfection supernatants (3 ml) were collected. Cells were also collected, washed and resuspended in 100 µl of SDS loading buffer. Supernatant and cells samples (10 and 5 µl, respectively) were loaded in a in a 10% polyacrylamide gel by SDS-PAGE. Using these sample volumes, cell samples are enriched ~20 times compared to supernatant samples. After electrophoresis, samples were blotted onto a nitrocellulose membrane, which was probed with a monoclonal antibody against GFP (panel A), a monoclonal antibody against Flag tag (panel B) or a monoclonal antibody against c-Myc tag (not shown). Bands were detected with an ECL kit.
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This analysis shows that both pGPI-PLD/EGFP-N3 and p3XFlag-CMV-8/GPI-PLD-transfected cells only secrete fusion proteins with a size corresponding to the predicted for each construct. Even GPI-PLD/GFP releases a protein fragment that corresponds to the nearly intact sequence of GFP (Figure 4.7A), indicating cleavage at the C-terminal end of the GPI-PLD/GFP fusion protein. This result supports the hypothesis that proteolytic cleavage at the carboxyl terminal end in the Myc-tagged GPI-PLD fusion protein results in its failure to be detected.

4.2.4 In vitro analysis of the activity of GPI-PLD fusion proteins.

The in vitro enzymatic activity of the different GPI-PLD fusion proteins was evaluated by the variant surface glycoprotein (VSG) degradation assay. VSG is a GPI-anchored glycoprotein expressed by African trypanosomes (Trypanosoma brucei) as a structural component of their surface coat (Turner et al., 1985). Myristic acid is the hydrophobic moiety responsible for the anchoring of VSG to the parasite membrane (Milne et al., 1999) (Figure 4.8A). To assay for GPI-PLD activity, H-labelled VSG was generated by incubating trypanosomes with [3H]-myristate (Ferguson and Cross, 1984). Purification of VSG from trypanosomes resulted in the generation of a homogeneous product with a molecular size of ~60 kDa. The protein concentration of the labelled VSG was 1 mg/ml with a specific activity of ~ 2000 cpm/µg (Figure 4.8B). The VSG degradation assay involves the incubation of labelled VSG in the presence of a source of GPI-PLD. After the incubation period, one of the reaction products, the [3H]-myristic acid, is recuperated by extraction with butanol and the organic phase is monitored for the amount of radioactivity. The suitability of the degradation assay to test the activity of GPI-PLD fusion proteins was evaluated by using mouse serum as GPI-PLD source. The results showed GPI-PLD activity in mouse serum in a dose-dependent manner (Figure 4.8C). This phospholipase activity was inhibited by addition of 1,10-phenanthroline, a known inhibitor of GPI-PLD activity (Low and Prasad, 1988).
Figure 4.8. Structure, synthesis and degradation of variant surface glycoprotein derived from trypanosomes. A) Scheme of trypanosome Variant Surface Glycoprotein (VSG) indicating the relative position of myristic acid within the chemical structure. The site where GPI-PLD exerts its hydrolytic activity is also shown. B) Metabolically-labelled VSG from trypanosomes. The purified sample was analysed by SDS-PAGE in a 12% gel. After running, the gel was stained with Coomassie blue and dried. C) VSG degradation by GPI-PLD present in mouse serum. The degradation assay was performed as indicated (Chapter 2, section 2.5.5.) and using serial dilutions of mouse serum. One reaction in the presence of the GPI-PLD inhibitor, 1,10-phenanthroline (Phen), was included. Data are means ± standard deviation of one experiment performed in duplicate. The result shown is representative of two independent experiments.
Figure 4.9 shows the activity of the different GPI-PLD fusion proteins produced in COS-7 on the cleavage of VSG. Only GPI-PLD expressed by the pcDNA3/GPI-PLD plasmid and, to a lower degree, the GPI-PLD/GFP fusion protein possessed enzymatic activity capable of degrading the VSG substrate.

![Graph showing activity of recombinant GPI-PLD](image)

**Figure 4.9** In vitro activity of recombinant GPI-PLD. Cell extracts of COS-7 transfected with the different GPI-PLD expression plasmids were tested for GPI-PLD enzymatic activity in a GPI-anchor degradation assay using the Variant Surface Glycoprotein (VSG) derived from trypanosomes as a substrate (white bars). 48 h after transfection, cells were washed and lysed by vortex mixing in a NP-40 buffer. The degradation reaction was started by the addition of reaction buffer containing the VSG substrate to the cell extracts. After 1 h at 37°C, the reaction was stopped by addition of NH₄OH-saturated butanol. The organic phase was separated by centrifugation, mixed with scintillation fluid and analysed by liquid-scintillation counting. 2 μl of mouse serum (MS) was used as positive control. The inhibitor 1,10-phenanthroline (1mM) was included in degradation reactions (black bars). Data are means ± standard deviation of one experiment performed in duplicate. The result shown is representative of two independent experiments.
In this assay the phospholipase activity was mediated by recombinant GPI-PLD because it could be inhibited by 1,10-phenanthroline, when it was included in the reaction mixture. The specificity of the reaction is also corroborated by the lack of enzymatic activity in samples derived from cells transfected with control plasmids coding for GFP. No VSG-degrading activity was detected in the cell extracts derived from cells transfected either with the pcDNA3/GPI-PLD/Myc-His A or the p3XFlag-CMV-8/GPI-PLD.

4.3 DISCUSSION

4.3.1 Expression of GPI-PLD fusion proteins

The analysis of the expression of Flag/GPI-PLD and GPI-PLD/GFP fusion proteins revealed the presence of additional bands to those predicted by the cDNA sequences (Figures 4.5 and 4.7). Analysis of the GPI-PLD protein sequence showed the presence of ten potential sites of N-glycosylation (Chapter 3, Table 3.1). It is possible that bands close to the expected molecular size are the result of a variable glycosylation site occupancy (Devasahayam et al., 1999). Supporting this, similar results have been obtained when comparing the expression of human GPI-PLD in mammalian CHO and insect H5 cells. The difference in the molecular weight of the GPI-PLD expressed in these two systems was attributed to different post-translational modifications, mainly sugar residues addition (Tsuijoka et al., 1998). It has also been demonstrated that recombinant and serum-purified GPI-PLD samples with a different molecular weight (100 and 115 kDa, respectively) show a similar molecular mass after N-glycosidase treatment, pointing to differences in the glycosylation pattern depending on the source of GPI-PLD (Stadelmann et al., 1997).
The detection of shorter forms of the GFP and Flag–tagged GPI-PLD, however, could also be the result of proteolytic cleavage. The presence of naturally occurring shorter forms of GPI-PLD in purified samples from bovine serum has been previously reported (Hoener et al., 1994). In addition, two independent reports have shown that treatment of bovine GPI-PLD with trypsin results in the generation of three fragments, which probably could represent functional domains of GPI-PLD (Heller et al., 1994; Hoener et al., 1994; Li et al., 1994). These results suggest the presence of natural protease-sensitive sites.

Analysis of the mouse GPI-PLD sequence reveals the presence of regions similar to those recognised by trypsin on the bovine sequence. Considering that the Flag tag is located at the amino end of the Flag/GPI-PLD fusion protein, single cleavage by endogenous proteases around the residues 330 or 590 on the mouse GPI-PLD would explain the detection of GPI-PLD fragments of ~40 and ~80 kDa observed in COS-7 cells (Figure 4.5A).

Cleavage at position 590 would explain the presence of a ~60 kDa band in extracts of COS-7 cells transfected with the pGPI-PLD/EGFP-N3 plasmid (Figure 4.5B). Cleavage at some other positions within GPI-PLD, however, would explain the presence of the other bands detected, in addition to the 140-kDa band predicted by the plasmid sequence. The detection of a protein fragment of similar size to GFP indicates that cleavage at the carboxyl terminal end, which results in the release of the GFP tag, is occurring in the transfected cells.

The detection of additional bands to those predicted by the plasmid sequences and the observation that some of them are detected at the same molar ratio over the time (at least in COS-7 cells transfected with p3XFlag-CMV-8/GPI-PLD plasmid, Figure 4.5A) would suggest that cleavage of GPI-PLD by endogenous proteases is a natural and specific process leading to the production of shorter GPI-PLD forms. However, as this
processing of GPI-PLD is not seen in other cell types, it could be possible that this pattern of bands is just specific for COS-7 cell which contain a high concentration of proteolytic enzymes.

It has been reported that \textit{in vitro} cleavage of GPI-PLD with trypsin does not modify neither its activity nor its requirement for detergents in order to cleave the GPI-anchored proteins on intact membranes (Li \textit{et al.}, 1994; Heller \textit{et al.}, 1994). For this reason, it is not known whether the \textit{in vivo} proteolytic process has implications for the functional roles of GPI-PLD. It would be interesting to determine which are the specific enzyme(s) that cleave GPI-PLD, and the intracellular site where this takes place.

\subsection*{4.3.2 Release of GPI-PLD into the medium}

As expected and because GPI-PLD contains a signal sequence, the release of GPI-PLD by transfected COS-7 cells has been confirmed in this work (Figure 4.7). This result is in agreement with previous observations on the release of GPI-PLD by cultured keratinocytes, myeloid, hepatic and pancreatic cells (Metz \textit{et al.}, 1991; Xie and Low, 1994; Deeg and Verchere, 1997).

Only one band was detected both in COS-7 cells transfected either with the pGPI-PLD/EGFP-N3 or the p3XFlag-CMV-8/GPI-PLD (Figure 4.6) plasmids. The detected band in the supernatants shows a slightly higher molecular weight than those observed the cell extracts, indicating a modification of the polysaccharides on GPI-PLD.

Similar results have been obtained when analysing the expression of transfected human GPI-PLD in CHO cells. In this work it was described that GPI-PLD was initially synthesised as a 105-kDa precursor which undergoes oligosaccharide processing resulting in the production of a mature 115-kDa form that is released into the medium. In this work it was also shown that the expression of GPI-PLD in insect cells results in
the synthesis of an incompletely processed GPI-PLD form of ~98 kDa (Tsujioka et al., 1998). The addition of complex-type sugars has profound effects on the recognition by antibodies because only non glycosylated forms (derived from insect cells) are recognised by an anti-C-terminal peptide antibody. The sugar processing is also related with modifications of the activity towards GPI substrates. In an in vitro assay, although both non- and fully-glycosylated GPI-PLD forms have the same activity in the presence of detergents, only the immature form was able to release a GPI-anchored protein from intact membranes (Tsujioka et al., 1998).

4.3.3 Enzymatic activity of GPI-PLD fusion proteins

The enzymatic activity of the fusion proteins encoded for the plasmid constructs designed in this work was evaluated by the in vitro VSG degradation assay (Figure 4.8). Only the pcDNA3/GPI-PLD and the GPI-PLD/GFP fusion proteins showed an enzymatic activity that when compared to the GPI-PLD activity present in serum was relatively lower (Figure 4.9). It is possible that this difference is due to the relative amount of GPI-PLD in serum and cell extracts samples. The GPI-PLD fusion proteins with epitope tags associated with the N-terminal (Flag/GPI-PLD) or C-terminal (GPI-PLD/Myc) ends were unable to cleave the GPI-anchored VSG, even in the presence of detergent (Figure 4.9).

In a previous study Hoener et al., demonstrated that GPI-PLD is associated in serum with apolipoprotein A-I (apoA-I), while purified GPI-PLD exists as an inactive aggregate (Hoener et al., 1993). These GPI-PLD aggregates become active after association with apoA-I, and in this way, only the complex GPI-PLD/apoA-I showed an efficient degrading activity of the GPI-anchored substrate acetylcholinesterase (Hoener et al., 1993). The results obtained with the pcDNA3/GPI-PLD and GPI-PLD/GFP fusion proteins could be related to these observations. Considering that plasma apolipoproteins consist of amphipathic alpha-helices (Davis, 1991) and that activation of GPI-PLD takes place
in the presence of an excess of apoA-I, it seems likely that apoA-I exerts a detergent-like action on GPI-PLD.

The addition of detergent in the VSG-degradation assays allows detection of the degrading activity of the pcDNA3/GPI-PLD and GPI-PLD/GFP fusion proteins. However, it is possible that the activity of the GPI-PLD fusion proteins could be further enhanced by the presence of apoA-I. These results suggest that apoA-I could play an important role in the regulation of GPI-PLD activity in vivo. It would be interesting to determine the mechanisms of association-dissociation of the GPI-PLD/apo A-I complexes and the way these complexes are internalised by the cells.

That no protein was detected with the GPI-PLD/Myc fusion protein is probably related with the previously mentioned inability to detect the production of any protein encoded by the pcDNA3/GPI-PLD/Myc-His A plasmid. Stadelmann et al., have demonstrated that the C-terminal end of GPI-PLD is essential for the enzymatic activity (Stadelmann et al., 1997). Based on that study, and if the lack of detection of this protein is attributed to the cleavage of the fusion protein at the C-terminal end (which results in the release of the Myc tag), then this probably would be also affecting its activity. In the case of p3XFlag-CMV-8/GPI-PLD, even when the protein is produced by the transfected cells (Figures 4.5 and 4.6), it is possible that the addition of the Flag epitope tag inhibits the enzymatic properties of GPI-PLD. Flag epitope sequence contains glutamic acid, which probably create a highly charged N-terminus that may affect the enzymatic activity of GPI-PLD. Alternatively, it could be possible that the enzymatic activity is reduced to a level not detectable by this assay.

In summary, in this work several plasmid constructs encoding for tagged-GPI-PLD fusion proteins have been designed. The fusion proteins are expressed and released by different cell lines after transient transfection and two of them (pcDNA3/GPI-PLD and GPI-PLD/GFP) showed enzymatic activity towards the GPI-anchored VSG
substrate in an *in vitro* assay. These results indicate that these plasmid constructs can be used to analyse the intracellular localisation of GPI-PLD and its association with other proteins.
CHAPTER 5

ANALYSIS OF GPI-PLD ACTIVITY IN VIVO

5.1 INTRODUCTION

GPI-anchored proteins constitute a diverse group of molecules involved in many different cell functions. The group includes growth factor receptors, adhesion molecules, hydrolytic enzymes and complement molecules. These molecules are widely distributed in eukaryotes and so far, over 150 different GPI-anchored molecules have been described (Ferguson and Williams, 1988).

It has been suggested that GPI-anchors confer functional advantages over transmembrane proteins. Low turnover rates, increased lateral mobility, and sorting to the apical surface of polarised cells are some of the characteristics of these proteins. Another important function of GPI anchors is to allow the cleavage by specific enzymes resulting in the release of potential second messengers and soluble glycoproteins from the cell surface (Horejsi et al., 1999; Jones et al., 1997). Supporting this hypothesis, the presence of soluble forms of GPI-anchored proteins has been demonstrated (Almqvist and Carlsson, 1988; Gennarini et al., 1984; Klemens et al., 1990; Medof et al., 1987; Nemoto et al., 1996; Wilhelm et al., 1999).

The mechanisms that control the release or shedding of GPI-anchored proteins might then have a great biological significance because released GPI-anchored proteins have demonstrated regulatory roles. For instance, recent studies have shown that soluble CD14 (the GPI-anchored receptor for bacterial lipopolysaccharide, LPS) (Haziot et al., 1988) regulates the activation and function of human T cell clones and mononuclear cells by inducing an inhibition of interleukin-2 (IL-2) and other cytokines such as IFN-γ and IL-4 (Rey Nores et al., 1999). The regulatory role of soluble CD14 has been also demonstrated in monocyte-like cell lines and B lymphocytes where it
can regulate the proliferation, differentiation and production of specific cytokines and immunoglobulin isotypes (Arias et al., 2000; Filipp et al., 2001; Labeta et al., 1993). In the particular case of CD14, two different soluble forms of the protein have been described (Labeta et al., 1993). One of them is a 55-kDa form lacking the GPI-anchor which is presumably produced as result of an alternative splicing. The other soluble form is derived from cleavage of the GPI-anchored membrane form and even though this cleavage has been characterised in some extent, the actual identity of the enzyme responsible is not known (Bazil and Stromminger, 1991; Bufler et al., 1995).

Some other studies have also shown the relevant regulatory role of soluble GPI-anchored proteins. In one of them, it was shown that the GPI-anchored heparan sulphate proteoglycan expressed in the surface of bone marrow stromal cells could be released after binding its ligand, the basic fibroblast growth factor (bFGF). The active bFGF/proteoglycan complex released then stimulated the proliferation of bone marrow stromal and stem cells (Brunner et al., 1994). In another study it was demonstrated that an ADP-ribosyltransferase (a GPI-anchored enzyme that transfers ADP-ribose to cell surface proteins resulting in inhibition of cytotoxic and proliferative activity) was specifically released as an active form from the surface of cytotoxic T cells after activation (Nemoto et al., 1996; Wang et al., 1994). It has been also demonstrated that the GPI-anchored multifunctional cell surface receptor for the serine proteinase, urokinase plasminogen activator (uPAR), can mimic or antagonise cell surface uPAR in regulating cell proliferation, adhesion and migration, the activities normally regulated by this receptor (Chavakis et al., 1988; Mizukami et al., 1988; Rao et al., 1995).

The analysis of the enzymatic products, the use of specific inhibitors, and GPI-PLD antisense mRNA have given evidence of the involvement of GPI-PLD in the generation of the soluble forms of GPI-anchored molecules (Brunner et al., 1994; Nemoto et al., 1996; Wilhelm et al., 1999).
The mechanism of GPI-anchored protein cleavage, however, has not been established and conclusive evidence for the physiological role of GPI-PLD has not been achieved. Studies where GPI-PLD and GPI-anchored proteins are expressed simultaneously in co-transfected cells (Bemasconi et al., 1996; Kung et al., 1997; Scallon et al., 1991) suggest that GPI-PLD action occurs inside the cells, within compartments where GPI-PLD (either endogenously produced or taken up from the media) and its substrates are encountered in membrane domains believed to be different from those on the cell surface (Metz et al., 1994).

Considering all this information, in this work several experiments were done with the aim of investigating the possible role of GPI-PLD on the release of GPI-anchored molecules. In first place, using the plasmid constructs coding for GPI-PLD fusion proteins (described in previous chapters of this work) and a GPI-anchored protein, placental alkaline phosphatase (PLAP), the activity of GPI-PLD was evaluated in co-transfection experiments. Secondly, by using a mouse model for the release of GPI-anchored proteins, the possible participation of GPI-PLD in this process was evaluated.
5.2 RESULTS

5.2.1 GPI-PLD activity

The functional characterisation of GPI-PLD fusion proteins encoded by different plasmid constructs designed in this work (Chapter 4) demonstrated that they could degrade the trypanosome-derived GPI-anchored protein VSG. These plasmids could therefore be used to assess the activity of GPI-PLD in vivo, by transfection of the expression plasmids into mammalian cells together with a plasmid coding for placental alkaline phosphatase (PLAP, a GPI-anchored protein and therefore a potential target of GPI-PLD). The cleavage of PLAP was quantified by measuring in a colorimetric assay the PLAP activity in supernatants and that associated with cells. Thus if PLAP activity is found in the supernatants of cells co-transfected with GPI-PLD coding plasmids this is due to the GPI-PLD-mediated cleavage of the GPI-anchor of PLAP.

As seen in Figure 5.1, a GPI-degrading activity was detected only with the GPI-PLD proteins encoded by the pcDNA3/GPI-PLD and pGPI-PLD/EGFP-N3 plasmids. This degrading activity is GPI-PLD-specific because no PLAP was released in cells transfected with a plasmids coding for PLAP and GFP. In the same way, no GPI-degrading activity was detected in cells transfected only with the PLAP-coding plasmid. No GPI-degrading activity was detected with the GPI-PLD/Myc and Flag/GPI-PLD fusion proteins, consistent with the lack of in vitro activity (Chapter 4).
Figure 5.1. GPI-PLD activity in transfected cells. COS-7 cells grown in medium supplemented with alkali-treated foetal calf serum (FCS) were co-transfected by the DEAE-dextran/chloroquine method. Co-transfections were done by using combinations of the indicated GPI-PLD-encoding plasmids and the pSV40-PLAP plasmid. Control co-transfection experiments with plasmids pSV40-PLAP and pEGFP-N3 (coding for GFP), and non-transfected cells (NT) were included. 48 h after transfection cells and supernatants were collected. Cells were trypsinised, washed with cold PBS and lysed by sonication in 200 µl of MES buffer. 5 µl of cell extracts and 50 µl of supernatants were assayed for PLAP activity in a final volume of 200 µl of reaction buffer. The OD was measured at 405 nm after 10 min of starting the reaction. The graph shows the ratio supernatant/cell extract (SN/CE) for each transfection. The results shown are representative of three independent transfection experiments where every sample was evaluated by triplicate.
5.2.2 *De-novo* synthesised GPI-PLD is responsible of GPI-degrading activity

The observation that GPI-PLD can be taken up from serum present in culture media, where it remains active inside the cells in lysosomal vesicles, has led to one proposed mechanism of action for GPI-PLD (Hari et al., 1997; Hari et al., 1996). In this mechanism, GPI-PLD taken up from extracellular fluids is directed to lysosomal vesicles where it could interact with its substrates resulting in the cleavage of the GPI anchors. However, the co-transfection experiments presented above and similar studies with bovine and human GPI-PLD (Kung et al., 1997; Scallon et al., 1992) suggest that *de-novo* synthesised-GPI-PLD is responsible for the cleavage of GPI-anchored proteins. This possibility was further evaluated by co-transfection experiments using a plasmid coding for an antisense GPI-PLD, which would be able to block the *de novo* synthesis of GPI-PLD and therefore the enzymatic activity.

An antisense GPI-PLD plasmid (pcDNA3/asGPI-PLD/GFP) was constructed as outlined in Figure 5.2. The 5' end 1571 bp Sal I/Bam HI fragment obtained from pSPORT/GPI-PLD was inserted into Bam HI/Xho I sites of the pHRCMV-IR viral plasmid containing an internal ribosome entry sequence and GFP (Zufferey et al., 1997). This ligation resulted in the GPI-PLD fragment being placed in an inverse orientation (antisense). The 3 kb bicistronic construct was subcloned from pHRCMV-IR/asGPI-PLD into pcDNA3 (Figure 5.2B).

When this plasmid is transfected into mammalian cells, those taking up the plasmid DNA can be visualised by the GFP produced, thus the transfection efficiency can be monitored with GFP.
A) GPI-PLD Sal I/Bam HI fragment obtained after digestion of the pSPORT1/GPI-PLD plasmid was cloned into the pHRCMV-IR viral vector. This plasmid (pHRCMV-IR/asGPI-PLD), was digested with Bam HI and Not I and the fragment containing the antisense GPI-PLD, the IRES and the GFP sequences was cloned into pcDNA3 plasmid.

B) Schematic representation of pcDNA3/asGPI-PLD plasmid.

Figure 5.2. Diagram of the strategy followed to construct the pcDNA3/asGPI-PLD plasmid. A) A GPI-PLD Sal I/Bam HI fragment obtained after digestion of the pSPORT1/GPI-PLD plasmid was cloned into the pHRCMV-IR viral vector. This plasmid (pHRCMV-IR/asGPI-PLD), was digested with Bam HI and Not I and the fragment containing the antisense GPI-PLD, the IRES and the GFP sequences was cloned into pcDNA3 plasmid. B) Schematic representation of pcDNA3/asGPI-PLD plasmid.
Figure 5.3 shows the transfection of pcDNA3/asGPI-PLD into 293T, BHK and COS-7 cells. The expressed GFP showed an intense fluorescence concentrated in the nucleus as observed in cells transfected with the plasmid coding for GFP alone.

Figure 5.3. Expression of antisense GPI-PLD. Direct green fluorescence in living 293T (A), BHK (B) or COS-7 (C) cells 48 h after transfection with the pcDNA3/asGPI-PLD plasmid. No fluorescence was detected in control 293T, BHK and COS cells transfected with the pcDNA3 plasmid (D-F). These pictures are shown together with the phase microscope images (G-I).
The transfection efficiency of pcDNA3/asGPI-PLD was reasonably high, so it could be used to evaluate the effect of GPI-PLD on the release of PLAP. In Figure 5.4 it is shown that co-transfection with plasmids coding for GPI-PLD, PLAP and antisense GPI-PLD resulted in ~50% decrease of PLAP released into the medium if compared to cells transfected only with plasmids coding for GPI-PLD and PLAP. Control experiments with cells transfected with individual plasmids showed no PLAP in the supernatants. These results indicate that de-novo GPI-PLD expression is required for the cleavage of PLAP.

![Graph](image)

**Figure 5.4.** Endogenous GPI-PLD is responsible of PLAP release. 293T cells were co-transfected with plasmids pcDNA3/GPI-PLD, pSV40-PLAP and/or pcDNA3/asGPI-PLD as indicated. Cells were grown and transfected using a culture medium containing alkali-treated FCS. The alkaline treatment destroys the GPI-PLD activity present in serum. 48 h after transfection, supernatants and cells were collected. Processing of cells and quantification of PLAP activity in supernatants (□) and cell extracts (■) was done exactly as described in Figure 5.1. The results shown are the media +/- SD of triplicate measurements for each transfection condition in three independent experiments. Two-sample t tests were performed. * P<0.05.
As for these experiments, serum used in culture media was subjected to an alkaline treatment (to eliminate the GPI-PLD activity) the results also indicate that GPI-anchor cleavage is catalysed by cellular GPI-PLD rather than by serum GPI-PLD.

5.2.3. Modulation of GPI-PLD activity.

Once it was confirmed that endogenous GPI-PLD is responsible for the release of co-transfected GPI-anchored proteins, experiments were done in order to determine whether or not in vivo GPI-PLD activity can be modulated. Optimisation of transfection experiments showed that 293T cells were most efficiently transfected and contained the least proteolytic degradation activity (Figure 4.5). The 293T cells were therefore selected to study the modulation of GPI-PLD activity.

![Figure 5.5. Release of PLAP by GPI-PLD is inhibited by treatment with PMA. 293T cells were co-transfected with plasmids pGPI-PLD/EGFP-N3 (GPI-PLD) and pSV40-PLAP (PLAP). Cells were grown and transfected using a culture medium containing pH-treated FBS. The alkaline treatment destroys the GPI-PLD activity present in serum. 48 h after transfection, cells were washed twice with culture medium without serum and then incubated for 2 h in the presence of fresh medium containing 20 ng/ml of PMA. After the incubation period supernatants (□) and cells (■) were collected and analysed for PLAP activity. The results shown are the media +/- SD of triplicate measurements for each transfection condition in three independent experiments. Two-sample t tests were performed. * P<0.05.](image)
In these experiments pGPI-PLD/EGFP-N3 and pSV40/PLAP plasmids were co-transfected, and 48 h post-transfection cells were incubated for 2 h in the presence of 20 ng/ml of phorbol-12-myristate-13 acetate (PMA). After the stimulation period, cells and supernatants were collected and analysed for PLAP activity. The results in Figure 5.5 show that stimulation of co-transfected cells with PMA for 2 h inhibited the cleavage of PLAP by GPI-PLD if compared to non-stimulated cells.

5.2.4 GPI-PLD and release of GPI-anchored proteins.

Because of its hydrolysing activity, GPI-PLD has been postulated as an important regulator of the expression of GPI-anchored proteins. In this work this possibility was evaluated by analysing the release of CD14 (the GPI-anchored receptor for LPS) from a macrophage cell line.

Delgado et al., showed recently that two pleiotropic neuropeptides with anti-inflammatory effects, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) induced the release of CD14 from LPS-stimulated peritoneal macrophages (Bellinger et al., 1996; Delgado et al., 1999a; Garrido et al., 1996). In this study it was suggested that VIP and PACAP down-regulated membrane CD14 expression by inducing its rapid shedding. However, the identity of the enzyme(s) involved was not established.

Since GPI-PLD is expressed in macrophages, it was decided to determine if this enzyme could be involved in this process. For this, the mouse monocyte/macrophage cell line (RAW264.7), which does express GPI-PLD (Xie and Low, 1994), was stimulated with VIP and LPS for 2 h. The presence of CD14 in cell extracts and supernatants was analysed by Western blotting. The results obtained showed that a 55-kDa CD14 membrane form is detected in cell extracts of RAW264.7 (Figure 5.6A),
but in opposition to what was anticipated, no decrease in the level of membrane CD14 in extracts derived from cells treated with LPS and VIP was seen. The CD14 expression level is very similar to that of cells treated either with LPS or VIP alone. In the same way, no soluble CD14 was detected in the supernatants of cells under the same treatment (Figure 5.6B). Based on the observations of Delgado et al. (Delgado et al., 1999a), it was expected to detect soluble CD14 in the supernatants of cells stimulated with LPS and VIP.

Figure 5.6. Release of CD14 from stimulated macrophages. RAW264.7 macrophages (2x10^6 cells/well) were plated in 6-well plates and incubated for 2 h to allow adherence. After two washings with culture medium to eliminate non-adherent cells, monolayers were incubated at 37 °C in the presence of LPS, VIP or combinations of these two molecules. After 2 h supernatants were collected and cells were lysed and sonicated in 1X SDS loading buffer. Samples were resolved by SDS-PAGE in 12.5% acrylamide gels. After running the gels, cell extracts (A) and supernatants (B) samples were blotted onto nitrocellulose membranes and probed with an anti-mouse CD14 rat antibody and a goat anti-rat HRP-conjugated antibody. Immunoreactive bands were detected with reagents of an ECL kit. Results shown are representative of two independent experiments, where similar results were obtained.
It has been well established that cleavage of GPI-anchored protein results in the generation of soluble proteins that no longer associate with the membrane. It has been proposed recently, however, that cleavage of GPI-anchors can also result in conformational changes in the protein that affect its ability to be recognised by specific antibodies (Butikofer et al., 2001). Considering this as a possible cause of the inability to detect any soluble CD14 in the previous experiments, it was decided to re-analyse the effect of VIP on macrophages using a “sandwich” ELISA technique, which is a more sensitive technique than Western blot. This was done also considering the possibility that if released, CD14 would be present in the supernatants in a very low concentration (Figure 5.7).

**Figure 5.7.** (A) ELISA evaluation of CD14 release by stimulated macrophages. RAW264.7 macrophages were plated in 96-well plates (2.5x10⁵ cells/well) and incubated for 2 h to allow adherence. Cells were incubated at 37°C in the presence of VIP (100 nM), LPS (10 ng/ml) or a combination of the two inducers. After 2 h of stimulation, supernatants were collected and analysed for the presence of soluble CD14 by a “sandwich” ELISA technique. A schematic diagram of the antibodies used for detection of CD14 is shown (B). The results shown are the mean ± the standard deviation of an experiment representative of three independent experiments performed in triplicate.
As seen in Figure 5.7A, there are no significant differences in the level of CD14 in supernatants in cells treated with VIP and LPS compared to non-stimulated cells or cells incubated either with LPS or VIP alone. Delgado et al. found a significant increase of soluble CD14 in the supernatants of macrophages incubated with LPS together with VIP (Delgado et al., 1999a), however, it was not possible to reproduce these results, nor was it possible to find any shedding of CD14 in peritoneal macrophages incubated with LPS and VIP.

As it has been well established that macrophages respond to LPS activation by releasing cytokines such as TNFα, IL-1, and IL-6 (Ulevitch and Tobias, 1995), and that VIP can down-modulate the effects of LPS by acting as a macrophage de-activating agent (Delgado et al., 1998; Delgado et al., 1999b). To ensure that RAW264.7 cells were responsive to LPS and VIP treatment, it was decided to analyse the production of TNFα by cell stimulated with LPS and VIP.

As shown in Figure 5.8A, RAW264.7 cells incubated with LPS produced and secreted TNFα into the medium. VIP by itself had no effect on the production of TNFα, as compared to non-stimulated cells (Figure 5.8A). However, LPS failed to induce TNFα production when it was added to the cells together with VIP, confirming its macrophage de-activating function. These results show clearly that both LPS and VIP have the expected biological function in RAW264.7 cells.
Figure 5.8. ELISA evaluation of TNFα production by stimulated macrophages. RAW267.4 macrophages were plated in 96-well plates (2.5X10^5 cells/well) and incubated for 2 h to allow adherence. Cells were incubated at 37°C in the presence of VIP (100 nM), LPS (10 ng/ml) or a combination of the two inducers. After 6 h of stimulation, supernatants were collected and analysed for the presence of TNFα by a “sandwich” ELISA technique (A). Schematic diagram of the antibodies used for detection of TNFα is shown (B). The results shown are the mean ± the standard deviation of one experiment representative of three independent experiments performed in triplicate.

5.3 DISCUSSION

The characterisation of GPI-PLD activity has shown that this enzyme cleaves detergent-solubilised GPI-anchored proteins and that this occurs only in the presence of detergents (Low and Huang, 1991). Because of its enzymatic activity, GPI-PLD has been proposed as a potential regulator of both the expression of GPI-anchored proteins and signal transduction pathways involving IPGs. According with these suggestions, knowing the precise mechanism and the site where GPI-PLD acts has become an important field of study that has received attention in the last years.
CHAPTER 5. ANALYSIS OF GPI-PLD ACTIVITY IN VIVO.

Certain studies have suggested that GPI-PLD is taken up from the extracellular fluids and directed to intracellular vesicles, most probably lysosomes, where it remains stable. According to this model, it would be in these lysosomal vesicles where GPI-PLD encounters and cleaves its substrates, resulting in the release of soluble forms of GPI-anchored proteins (Hari et al., 1997; Hari et al., 1996).

Other studies, however, have suggested that it is an endogenous GPI-PLD the responsible of cleavage of GPI-anchored proteins, and not that derived from the extracellular medium. Studies on tumour cells expressing high levels of the urokinase receptor (uPAR) (Wilhelm et al., 1999) and co-transfection experiments of GPI-PLD and GPI-anchored proteins (Bemasconi et al., 1996; Kung et al., 1997; Scallon et al., 1991) have provided evidence to support the idea that is the intracellular GPI-PLD responsible for GPI anchors cleavage.

In this work, I demonstrated that two plasmid constructs (pcDNA3/GPI-PLD and pGPI-PLD/EGFP-N3) coding for mouse GPI-PLD catalysed the release of PLAP, in co-transfection experiments. These results are additional evidence of the role of de-novo synthesised GPI-PLD in the release of GPI-anchored proteins. Further evidence supporting this idea was obtained in experiments of stimulation with PMA. As it has been reported that PMA stimulates the release of GPI-PLD in myeloid cells (Xie and Low, 1994), the same effect would explain the reduction of PLAP activity in supernatants of GPI-PLD/PLAP co-transfected cells. As GPI-PLD is been secreted from the cell, it would no longer be able to cleave the GPI-anchor of PLAP. This result is in agreement with previous observations that recombinant GPI-PLD purified from media of transfected cells is not longer active on intact cells, even though it proved to be active towards solubilised substrates in in vitro experiments (Scallon et al., 1991). It is possible to speculate that the PMA-mediated release of GPI-PLD is induced by the activation of protein kinase C (PKC), which is activated in response to PMA stimulation (Visnjic et al., 1995).
Stronger evidence that de-novo GPI-PLD is responsible for cleavage of GPI-anchored proteins was obtained in co-transfection experiments using a plasmid coding for a GPI-PLD antisense fragment that resulted in the inhibition of the PLAP release. Taken together all these data indicate that cleavage of GPI-anchored proteins require the de-novo synthesis of both GPI-PLD and the GPI-anchored proteins.

Having established that GPI-PLD cleaves the GPI-anchored proteins resulting in the release of soluble GPI-anchored proteins, it was decided to evaluate this activity in a more physiological model, as transfection protocols normally induce a high expression of recombinant proteins that in most cases does not correlate with the expression level under normal conditions. For this reason cells normally expressing endogenous GPI-PLD and GPI-anchored proteins were used to evaluate the GPI-PLD activity after stimulation.

In previous studies evidence was given pointing to GPI-PLD being responsible for the release of two GPI-anchored molecules, decay accelerating factor (DAF) and the receptor complex for basic fibroblast growth factor (Brunner et al., 1994; Metz et al., 1994) from normal human bone marrow cells. In another study, it was shown that CD14, the GPI-anchored receptor for LPS, was released from mouse macrophages after stimulation with LPS and VIP, a potent neuropeptide with immunoregulatory properties. In this case, the mechanism or molecules involved were not identified. However, the observation that LPS and VIP stimulation did not affect the CD14 mRNA levels suggested the participation of phospholipases degrading the GPI-anchor (Delgado et al., 1999a).

To evaluate the possible participation of GPI-PLD in the CD14 shedding process, the macrophage cell line RAW267.4, expressing both GPI-PLD (Figure 3.15) and CD14 (Ziegler-Heitbrock et al., 1993), was selected. The results obtained, however, were not successful in detecting soluble CD14 after stimulation of RAW267.4 cells with LPS and
VIP. Different facts could explain this lack of reproducibility of what has been previously shown (Delgado et al., 1999a). First, the macrophage population used in this study, the RAW267.4 (monocyte/macrophage) cell line could be different, in terms of activation state, to the peritoneal macrophage population used by Delgado et al. It is possible that the thioglycollate-based protocol used to enrich and obtain the macrophage population makes these cells more susceptible to respond to the different stimuli, whilst the RAW267.4 monocyte/macrophages could be less efficient to respond to the same treatment. However, LPS-stimulation of RAW267.4 cells clearly showed that they are able to produce TNFα in a manner that could be regulated by VIP (Figure 5.8).

If the CD14 shedding is mediated by GPI-PLD, peritoneal macrophages in a more advanced differentiation state could be more efficient than RAW267.4. Supporting this idea is the observation that GPI-PLD activity is increased along with the myeloid cell differentiation (Xie and Low, 1994). Also supporting this is the observation that the release of endogenous PLAP in COS-1 cells was only detected after transfection with a plasmid coding for GPI-PLD, that increased the concentration of intracellular GPI-PLD (Kung et al., 1997). It could be also possible that peritoneal macrophages have taken up more GPI-PLD from the medium and therefore have a different activity to that of RAW267.4 cells.

Secondly, the methods used to detect soluble CD14 in this work are different to those used by Delgado et al. In that work, soluble CD14 was immunoprecipitated in supernatant samples from surface iodinated macrophages. This approach seems to be more powerful to detect small amounts of released CD14 compared to the Western blot analysis and ELISA employed in this work.
The inability to detect a GPI-PLD-mediated cleavage of GPI-anchored proteins in this work could be related to the fact that not a great number of GPI-anchored molecules are released as a result of GPI-PLD activity. Because of the potential regulatory roles of the soluble forms of GPI-anchored proteins, their release might be a highly regulated process.
CHAPTER 6
GPI-PLD INTRACELLULAR LOCALISATION

6.1 INTRODUCTION.

So far, over 150 different GPI-anchored proteins have been described in mammalian cells and their role in many different cellular functions has been well established (Marmor and Julius, 2000). It is believed that GPI anchors serve as substrates for the generation of second messengers when they are selectively cleaved by specific GPI-phospholipases (Jones et al., 1997).

GPI-PLD, the enzyme presumed to be responsible for the cleavage of GPI-anchored molecules, is present at relatively high concentration in normal serum (Davitz et al., 1987; Low and Prasad, 1988). It has been demonstrated that liver is the main tissue source of GPI-PLD, although most cell types express this enzyme at low levels (Chapter 3, this thesis). A direct demonstration, however, of GPI-PLD degrading activity on intact membranes has not been observed. The GPI-PLD enzymatic activity has been demonstrated only in the presence of detergents or cholesterol binding agents that perturb the structure of the cell membrane (Low and Huang, 1991; Bergman et al., 1994), or observed when GPI-PLD is co-expressed with GPI-anchored proteins (Chapter 5).

It has therefore been suggested that the catalytic activity of GPI-PLD occurs inside the cells, within compartments where GPI-PLD (either endogenously produced or taken up from the media) and its substrates are encountered in membrane domains believed to be different from those on the cell surface (Metz et al., 1994). The precise site where this occurs, however, remains unknown.

Results from several studies aiming to determine the GPI-PLD intracellular localisation suggest that this enzyme is localised in vesicles and membrane domains presumed to
be the Golgi system. It has been also proposed that GPI-PLD is localised within intracellular compartments of the secretory pathway, with the cleavage of GPI-anchored proteins occurring when they are being directed to the cell surface (Scallon et al., 1991; Bernasconi et al., 1996).

Considering that the precise intracellular site where GPI-PLD is expressed and exerts its activity is still controversial, it was decided to examine the subcellular localisation and evaluate the possibility of GPI-PLD being present within membrane domains termed caveolae. Several lines of evidence suggested such localisation. Firstly, it has been described that caveolae are enriched in GPI-anchored proteins, presumed to be the targets of GPI-PLD (Parton and Simmons, 1995). Secondly, analysis of the GPI-PLD protein sequence (see Chapter 3, Figure 3.4) revealed the presence of a sequence shared by proteins that interact with and are regulated by caveolins (Couet et al., 1997), which are structural components of caveolae (Kurzchalia et al., 1992; Rothberg et al., 1992). Finally, PLD2 one isoform of mammalian PLD has been found to be localised in caveolae (Czarny et al., 1999).

Based on these observations, the aim of the experiments presented in this chapter is to determine the intracellular localisation of GPI-PLD.
6.2. RESULTS

6.2.1 GPI-PLD staining pattern in different cell lines.

In Chapters 4 and 5 of this thesis it was demonstrated that mammalian cells transfected with the GPI-PLD plasmid constructs designed in this work expressed functional GPI-PLD fusion proteins. The presence of GFP or Flag tags facilitates the detection by direct fluorescence (GPI-PLD/GFP) or indirect immunofluorescence (Flag/GPI-PLD) therefore, it is possible to analyse the GPI-PLD intracellular expression and subcellular localisation in different cell lines by using these fusion proteins.

The experiments outlined in Chapter 4 demonstrated that the Flag/GPI-PLD fusion protein was detected by Western blot analysis in different cell lines (Figure 4.5 A-C), although it did not appear to have any enzymatic activity in the VSG-degradation assay (Figure 4.9), nor following co-transfection with PLAP in COS-7 cells (Figure 5.1). Despite the fact that it has been reported that epitope tags do not interfere with fusion protein expression and activity (Knappik, 1994; Molloy et al., 1994), it could be possible that the addition of three acidic/charged Flag (3XFlag) epitope sequences (Asp-Tyr-Lys-Asp-His-Asp) to the N terminus of GPI-PLD affects its activity and/or probably, its intracellular localisation.

In order to address this issue, the staining pattern of the Flag/GPI-PLD fusion protein was analysed and compared with that of the GPI-PLD/GFP in COS-7 cells co-transfected with pGPI-PLD/EGFP-N3 and the pCMV8-3XFlag/GPI-PLD plasmid constructs. Figure 6.1A and 6.1B show that GFP and Flag-tagged GPI-PLD have a similar fluorescence pattern. Merging of the fluorescence patterns confirmed that both fusion proteins had the same intracellular localisation (Figure 6.1C). This result demonstrates that GPI-PLD intracellular localisation is not affected by its expression as a tagged fusion protein and in particular, the charged Flag epitope did not alter the subcellular distribution of GPI-PLD. Based on this finding it was considered that
Figure 6.1. GFP and Flag-tagged GPI-PLD have the same intracellular localisation in COS-7 cells. COS-7 cells were transiently co-transfected by the DEAE-dextran/chloroquine method with the pGPI-PLD/EGFP-N3 and pCMV8-3XFlag/GPI-PLD plasmid constructs. 48 h after transfection cells were fixed with paraformaldehyde and permeabilised with Triton X-100. The Flag/GPI-PLD was stained with a primary mouse anti-Flag antibody and a secondary phycoerythrin-conjugated rabbit anti-mouse antibody. Pictures of green fluorescence corresponding to the GFP/GPI-PLD staining pattern (A) and red fluorescence corresponding to Flag/GPI-PLD (B), were merged resulting in a yellowish staining pattern (C). About 90% of transfected cells incorporated both plasmids. Over 50 double-transfected cells were analysed in randomly selected fields. Results are representative of two experiments where similar results were obtained.
although Flag/GPI-PLD fusion protein shows no detectable enzymatic activity, it is also suitable for use to analyse GPI-PLD intracellular localisation and association with other proteins.

The GPI-PLD subcellular localisation was analysed by microscopic analysis of 293T, COS-7, and BHK cell lines transiently-transfected with the plasmid constructs pGPI-PLD/EGFP-N3 or pCMV8-3XFlag/GPI-PLD. The fluorescence pattern of cells after 48 h of transfection is shown in Figure 6.2. Cells transfected with the plasmids coding for GPI-PLD fusion proteins, GPI-PLD/GFP (Figure 6.2 B, G, L) or Flag/GPI-PLD (Figure 6.2 C, H, M) show a fluorescence pattern where the recombinant protein is excluded from the cell nucleus but present in the perinuclear region. The staining patterns of GPI-PLD/GFP and Flag/GPI-PLD fusion proteins were similar in the three different cell lines tested.

The GPI-PLD fluorescence pattern was different to that observed when cells were transfected with a control plasmid containing only the GFP gene. In this case, the green fluorescence is highly concentrated in the nuclear region (Figure 6.2 A, F, K), corresponding to the fluorescence pattern reported for the GFP in transfected mammalian cells (Cubitt et al., 1995). No fluorescence was detected in control non-transfected cells (Figure 6.2 E, J, O).

6.2.2 GPI-PLD is not localised on the cell surface.

The fluorescence patterns observed in the three different cell lines showed that GPI-PLD is not expressed in the cell membrane. To corroborate this, permeabilised and non-permeabilised BHK cells transfected with the pCMV8-3XFlag/GPI-PLD were analysed. The lack of Triton X-100 treatment would allow the detection only of the Flag/GPI-PLD fusion protein if it was localised in the cell membrane.
Figure 6.2. Intracellular localisation of GPI-PLD in different cell types. 293T (A-E), COS-7 (F-J) and BHK (K-O) were transiently transfected with pEGFP-N3, pGPI-PLD/EGFP-N3, or pCMV8-3XFlag/GPI-PLD plasmid constructs (5 μg for each plasmid). After 48 h of transfection, cells transfected with the pEGFP-N3 (A, F, K) or pGPI-PLD/EGFP-N3 (B, G, L) plasmids were fixed. Cells transfected with the pCMV8-3XFlag/GPI-PLD plasmid were fixed with para-formaldehyde after transfection, permeabilised with Triton X-100 and stained by using a primary mouse anti-Flag antibody and a secondary FITC-conjugated rabbit anti-mouse antibody (C, F, I). Phase (D, I, N) and fluorescence (E, J, O) microscope pictures are also shown for control non-transfected cells stained using the same protocol as for cells transfected with the pCMV8-3XFlag/GPI-PLD plasmid. Size bars, 20 μm.
Non-transfected cells

COS-7

pEGFP-N3  pGPI-PLD/EGFP-N3  pCMV8-3XFlag/ GPI-PLD

BHK

pEGFP-N3  pGPI-PLD/EGFP-N3  pCMV8-3XFlag/ GPI-PLD

Non-transfected cells
Figure 6.3A shows the staining pattern of BHK cells treated with Triton X-100. As previously indicated (Figure 6.2), Flag/GPI-PLD is expressed in the perinuclear region. No staining was revealed in non-permeabilised transfected cells (Figure 6.3 C). This was similar to the staining observed in control cells incubated only with secondary FITC-conjugated antibody (not shown). These results suggest that GPI-PLD, is not localised at the cell membrane (compare staining pattern of gangliosides GM1 present in the cell membrane, Figure 6.3 E), however, they do not rule out the possibility that Flag epitopes on GPI-PLD are not accessible or that they are located in the cytosolic side.

6.2.3 GPI-PLD and caveolin-3 co-localisation.

In addition to its structural role in the formation of caveolae (Parton, 1996), caveolins are key regulators of signalling events by associating with and regulating the activity of many different signalling proteins (Okamoto et al., 1998). It has been also shown that caveolins are involved in the regulation of cholesterol transport from intracellular organelles to the cell surface (Fielding and Fielding, 1996). Most of the studies on the regulating roles of caveolin have focused on its plasma membrane role, but recent evidence suggest that caveolin has a functional role in intracellular compartments (Luetterforst et al., 1999).

To investigate the possible co-localisation of GPI-PLD and caveolin, two plasmids encoding for caveolin-3 (Cav-3, kindly provided by Dr. R. G. Parton, University of Queensland, Australia) were used. One of the plasmids encodes for the wild type form of Cav-3 (Cav-3 WT), and the other for a truncated form of Cav-3 (Cav-3 DGV). The truncated form (residues 54-151) lacks 53 amino acids of the N terminus but contains the conserved scaffolding domain and behaves as a dominant-negative mutant caveolin (Luetterforst et al., 1999).
Figure 6.3. Flag/GPI-PLD fusion protein is not expressed at the cell membrane. BHK cells were transfected with pCMV8-3XFlag/GPI-PLD. 48 h after transfection cells were fixed by paraformaldehyde treatment. Some cells were permeabilised by incubation with Triton X-100 (A), while others were left untreated (C and E). Flag/GPI-PLD fusion protein was detected by intracellular staining using a mouse anti-Flag monoclonal antibody and a rabbit anti-mouse FITC-conjugated antibody (A). Staining of cell membrane gangliosides was achieved by incubation of BHK cells with phycoerythrin-conjugated CTB (E). B and D show the phase microscope pictures of stained cells. Bar size, 20 µm.
It has been shown that the negative effect of Cav-3 DGV results from interference with the cholesterol-regulatory role of caveolin (Roy et al., 1999). Both Cav-3 WT and Cav-3 DGV were cloned into the pCB6-KXHA plasmid, containing a haemagglutinin (HA) epitope tag (YPYDVPDYA), downstream of an in frame Not I site, thus these plasmids produce recombinant Cav-3 with a C-terminal HA tag (Luetterforst et al., 1999). For transfection experiments using these plasmids, BHK cells were selected because they lack endogenous Cav-3. This characteristic of BHK cells is very important as it has been demonstrated that caveolins can form homo-oligomers, which could potentially influence the distribution of introduced caveolins (Song et al., 1997).

Figure 6.4. Intracellular localisation of Cav-3 WT and Cav-3 DGV. Confocal pictures of BHK cells transiently transfected with 5 μg of plasmids coding either for the HA-tagged Cav-3 WT (A) or the dominant-negative HA-tagged Cav-3 DGV (B). 24 h after transfection, cells were fixed, permeabilised and stained using a primary mouse anti-HA monoclonal antibody and a FITC-conjugated rabbit antibody. Bar size, 20 μm.
Initially, the cellular localisation of Cav-3 WT and Cav-3 DGV was determined by intracellular staining of transfected BHK cells (Figure 6.4). As previously indicated, staining with antibodies against the C-terminal HA tag, revealed that Cav-3 WT localises to the cell surface, evenly distributed in complexes and to a perinuclear compartment assumed to represent the Golgi complex (Dupree et al., 1993; Luetterforst et al., 1999). In contrast, Cav-3 DGV showed no cell membrane staining but only perinuclear and punctate cytoplasmic staining in cell compartments that presumably represent the cis-Golgi (Luetterforst et al., 1999; Roy et al., 1999).

The possibility that GPI-PLD co-localises with caveolins in the same cell compartments was evaluated by intracellular staining and confocal microscopy analysis of BHK cells co-transfected with GPI-PLD/GFP and Cav-3. BHK cells co-transfected with pGPI-PLD/EGFP-N3 and the pCB6-KX/Cav-3 WT/HA plasmids showed no areas of strong co-localisation but weak yellowish perinuclear areas (indicated with white arrows) when the images of green and red fluorescence (GPI-PLD/GFP and Cav-3 WT, respectively) were merged (Figure 6.5B). However, no membrane co-localisation of Cav-3 WT and GPI-PLD was observed. Further analysis at different levels of the transfected cells did not show extensive co-localisation of these molecules (Figure 6.5B). This result suggests that the predominant amount of GPI-PLD does not co-localise with Cav-3 in the cell membrane compartments. However, if there is some co-localisation between these two molecules, this might occur not in membrane caveolae structures but in a different intracellular compartment.

Co-localisation of GPI-PLD and Cav-3, however, was evident when BHK cells co-transfected with pGPI-PLD/EGFP-N3 and pCB6-KX/Cav-3 DGV/HA plasmids were analysed (Figure 6.6A and B). In this case, yellow fluorescence areas are clearly seen when the images corresponding to the green and red fluorescence (GPI-PLD and Cav-3 DGV, respectively) were merged (Figure 6.6A).
Figure 6.5. Confocal microscopic analysis of GPI-PLD and Cav-3 WT co-transfected cells. BHK cells were co-transfected with the pGPI-PLD/EGFP-N3 (GPI-PLD) and pCB6KX/Cav-3 WT/HA (Cav-3 WT) plasmids. 24 h post-transfection cells were permeabilised by incubation with Triton X-100. Cav-3 WT was detected by indirect immunofluorescence after intracellular staining using a mouse anti-HA monoclonal antibody and a secondary phycoerythrin-conjugated rabbit anti-mouse antibody. GPI-PLD/GFP fusion protein was detected by direct fluorescence. A series of 7 pictures for each green (GPI-PLD) and red (Cav-3 WT) fluorescence taken at different levels every 0.2 μm is shown in A. The fourth picture of the series is enlarged and shown in B. Size bar, 20 μm.
Figure 6.6. Confocal microscopic analysis of GPI-PLD and Cav-3 DGV co-transfected cells. BHK cells were co-transfected with the pGPI-PLD/EGFP-N3 (GPI-PLD) and pCB6KX/Cav-3 DGV/HA (Cav-3 DGV) plasmids. 24 h post-transfection cells were permeabilised by incubation with Triton X-100. Cav-3 DGV was detected by indirect immunofluorescence after intracellular staining using a mouse anti-HA monoclonal antibody and a secondary phycoerythrin-conjugated rabbit anti-mouse antibody. GPI-PLD/GFP fusion protein was detected by direct fluorescence. A series of 7 pictures for each green (GPI-PLD) and red (Cav-3 WT) fluorescence taken at different levels every 0.2 μm is shown in A. The fourth picture of the series is enlarged and shown in B. Size bar, 20 μm.
CHAPTER 6. GPI-PLD INTRACELLULAR LOCALISATION.

The exclusion of this truncated dominant negative form of Cav-3 from the membrane and its concentration in other cell compartments, (presumably representing the cis-Golgi) (Luetterforst et al., 1999; Roy et al., 1999) may explain the bright intensity of the perinuclear yellow areas observed in these pictures. This result suggests that GPI-PLD and caveolin are localised in the same intracellular compartments, possibly related to cis-Golgi complex compartments. Additional experiments that could help to corroborate this suggestion would include the staining of GPI-PLD transfected cells with antibodies recognising proteins exclusively localised in cis-Golgi. Antibodies directed to the protein p23 (Rojo et al., 1997), a cis Golgi marker, or sialotransferase, a specific marker of the Trans-Golgi Network (TGN) (Rabouille et al., 1995), have been used to characterise the intracellular location of the Cav-3 DGV mutant in BHK cells (Luetterforst et al., 1999). Staining using these antibodies could give supporting evidence of a GPI-PLD localisation in Golgi-related compartments. Staining of 293T cells with a monoclonal antibody against galactosyltransferase-1 (Keusch et al., 1998) was tried in this thesis. Because of the weak fluorescence obtained with this antibody it was not possible to make the co-localisation analysis.

6.2.4 Subcellular fractionation over sucrose density gradients.

The confocal microscopy analysis of the co-expressed GPI-PLD and Cav-3 did not totally show unequivocal evidence of an association between these two molecules, therefore this question was further investigated by membrane fractionation using sucrose gradients. In these experiments transfected BHK cells were disrupted using a detergent-free procedure and the membranes separated using a sucrose density gradient (Song et al., 1996). Purification protocols to separate caveolin from the bulk of cellular membranes and cytosolic proteins take advantage of the specific buoyant density of caveolin-rich membrane domains and their resistance to solubilisation by non-ionic detergents such as Triton X-100 at low temperatures (Sargiacomo et al.,
1993). By using these purification protocols, it has been demonstrated that epitope-tagged Cav-3 behaves as the endogenous form in and is correctly targeted (Kurzchalia et al., 1992).

It has been suggested, however, that inclusion of detergent in the initial homogenisation step results in the loss of resident caveolae-associated proteins (Chang et al., 1994). To preserve these associations, a detergent-free method to purify caveolin-rich membrane domains was developed. This procedure replaces Triton X-100 detergent with sodium carbonate, which is routinely used to determine if proteins are firmly attached to membranes and does not solubilise caveolin. After homogenisation and sonication, the cell homogenate is separated by ultracentrifugation over a 5-35% sucrose density gradient and then fractions are collected and analysed by Western blotting (Song et al., 1996).

As previously described (Song et al., 1996; Roy et al., 1999), the subcellular fractionation showed that Cav-3 WT and Cav-3 DGV are present in the top fractions (3-4) of the sucrose density gradient (Figure 6.7B, D, F and H). Although most of the cellular GPI-PLD/GFP and Flag/GPI-PLD fusion proteins fractionated to the bottom fractions (7-9) of the gradient (Figure 6.7 A, C, E and G), a small fraction of the full-length polypeptide co-fractionated with Cav-3 WT in fraction number 4. This separation is more evident when the Flag/GPI-PLD and the Cav-3 DGV (Figure 6.7 G and H) plasmids are analysed. This result seems to contradict the confocal analysis results which showed an association of GPI-PLD within compartments shared by Cav-3 DGV. The subcellular fractionation analysis indicates that most of GPI-PLD is present in the bottom fractions (high density) of the gradient in a different compartment to Cav-3 DGV. This result could be explained considering that GPI-PLD, which has no membrane attachment, is located in the lumen of these Cav-3 DGV-enriched compartments.
There is the possibility that the GPI-PLD reactive bands detected in the caveolin-enriched fraction 4 of the sucrose gradient are due to contamination from the bottom fractions of the gradient. To discard this possibility and support the GPI-PLD distribution pattern within the sucrose gradient it would be necessary to probe the membranes with antibodies to proteins localised exclusively in endoplasmic reticulum membranes or other organelles which migrate to the high density fraction in a sucrose gradient. The detection of endoplasmic reticulum proteins only in the bottom fractions and not in the upper low-density fraction would indicate that no contamination whilst collection the different fractions of the gradient has occurred. One suitable protein for probing these membranes could be calnexin, which has been described as a resident of the endoplasmic reticulum (Wada et al., 1991).

As previously shown in this thesis (Figures 4.5 A and D), GFP or Flag-tagged GPI-PLD fusion proteins of different molecular weight can be observed by Western blot analysis of gradient fractions. One interesting point to note from the sucrose gradient analysis is that bands of low molecular weight are not separated into fraction 4 of the gradients. Only one band of high molecular weight was detected in this fraction, whilst the smaller molecular weight forms (probably proteolysis-derived fragments) were present in the bottom fractions of the gradient corresponding to endoplasmic reticulum and cytoplasm fractions. This result could indicate that total integrity of GPI-PLD, and possibly full glycosylation are important characteristics that determine its localisation is specific cell compartments.

Another indication that GPI-PLD might be associated or at least be in the same subcellular compartment with Cav-3 DGV was obtained from treatment of transfected cells with methyl-β-cyclo dextrin (MβCD), which depletes cholesterol from cell membranes (Furuchi and Anderson, 1998).
Figure 6.7. Subcellular fractionation over sucrose gradients of GPI-PLD and caveolin-3 WT and DGV co-transfected cells. BHK cells were co-transfected with pGPI-PLD/EGFP-N3, pCMV8-3XFlag/GPI-PLD, pCB6KX/Cav-3 WT/HA and pCB6KX/Cav-3 DGV/HA. 48 h post-transfection, cells were washed and homogenised in pH 11 sodium carbonate buffer. Cell lysates were
adjusted to 45% sucrose, placed in the bottom of centrifuge tubes and overlaid with equal volumes of 35% and 5% sucrose solutions. After 20 h of centrifugation, gradient fractions were collected and analysed by Western blotting. GPI-PLD was detected either with a mouse anti-GFP (A and C) or a mouse anti-Flag monoclonal antibodies (E and G). Cav-3 WT (B and D) and Cav-3 DGV (F and H) were detected with a mouse anti-HA monoclonal antibody.
BHk cells transfected with the pCMV8-3XFlag/GPI-PLD plasmid were incubated for 1 h in the presence of MβCD. As seen in Figure 6.8, MβCD induces dispersion in the staining pattern of transfected cells. This result probably means that the cell compartments where GPI-PLD was found to be co-localising with Cav-3 DGV are affected by MβCD-induced cholesterol depletion. However, a definitive conclusion cannot be drawn as control experiments for staining of other Golgi-resident and non-resident proteins should have been included. The analysis of the dispersion of proteins such as p23 and sialotransferase (Rojo et al., 1997; Rabouille et al., 1995) and comparison to that of GPI-PLD after treatment with MβCD could give stronger evidence of a co-localisation of GPI-PLD within the Golgi complex.

Figure 6.8. Cholesterol depletion affects the GPI-PLD staining pattern. BHK cells were transfected with the pCMV8-3XFlag plasmid. 48 h after transfection, culture medium was replaced for medium without serum and the cells were grown for 20 h at 37°C. After serum starvation, cells were incubated for 1 h with 2% MβCD. A) Control transfected cells not incubated with MβCD (B). After cholesterol depletion, the cells were washed, fixed and permeabilised with Triton X-100. Flag/GPI-PLD was stained with a mouse anti-Flag monoclonal antibody and a secondary FITC-conjugated rabbit anti-mouse IgG antibody. Bar size, 15 μm.
6.2.5 GPI-PLD does not co-localise with caveolae specific markers.

That GPI-PLD is not normally associated with the caveolae was confirmed by incubating transfected cells with cholera toxin subunit B (CTB) conjugated to a red fluorescent dye. CTB has a great affinity for the gangliosides GM1 and has been used as a marker of caveolae because these cell compartments are highly enriched in gangliosides GM1 (Kenworthy et al., 2000; Parton, 1994).

Detection of gangliosides GM1 with the fluorescent CTB probe showed a cell surface-like staining pattern in 293T cells transfected with the pGPI-PLD/EGFP-N3 plasmid. GPI-PLD/GFP fusion protein showed a perinuclear staining, and no co-localisation was observed when pictures for the red and green fluorescence were merged (Figure 6.9A and B).

When non-permeabilised transfected cells are incubated with CTB, this probe is accessible only to gangliosides GM1 at the cell surface. However, incubation of permeabilised transfected cells incubated with CTB would allow the detection of intracellular gangliosides GM1. When pCMV8-3XFlag/GPI-PLD-transfected 293T cells were incubated with the CTB probe after permeabilisation and intracellular staining of Flag/GPI-PLD fusion protein, certain areas of co-localisation of GPI-PLD and gangliosides GM1 were observed. That both GPI-PLD and gangliosides GM1 co-localise to the same compartments is supported by the confocal analysis, where the fluorescence intensity of the merged pictures is higher at only one level (Figure 6.10A and B). This co-localisation pattern can be explained by considering that gangliosides GM1 are present in endocytic organelles and the trans-Golgi network in addition to the plasma membrane (Parton, 1994).
Figure 6.9. GPI-PLD does not co-localise with specific markers of caveolae gangliosides GM1. 293T cells were transfected with the pGPI-PLD/EGFP-N3 plasmid. 48 h after transfection cells were fixed and stained for 10 min with cholera toxin subunit B (CTB). A series of 7 pictures for each green (GPI-PLD) and red (CTB) fluorescence taken at different levels every 0.2 μm is shown in A. The fourth picture of each series is enlarged and shown in B.
Figure 6.10. GPI-PLD co-localises with gangliosides GM1 in intracellular compartments. BHK cells were transfected with the pCMV8-3XFlag/GPI-PLD plasmid. 48 h after transfection cells were fixed, permeabilised with Triton X-100 and stained for gangliosides GM1 with the red fluorescent CTB probe and GPI-PLD with a mouse anti-Flag monoclonal antibody and a secondary FITC-conjugated anti-mouse rabbit antibody. Merging of the red and green fluorescence staining patterns shows yellow fluorescent areas of intracellular co-localisation. A series of 7 pictures for each green (GPI-PLD) and red (CTB) fluorescence taken at different levels every 0.2 μm is shown in A). The fourth picture of each series is enlarged and shown in B).
6.2.6 GPI-PLD is present in the Golgi complex

In order to obtain more evidence of a localisation for GPI-PLD in the Golgi complex, BHK cells transfected with the pCMV8-3X-Flag/GPI-PLD plasmid were treated with the microtubule-depolymerising agent nocodazole which disrupts the Golgi complex (Kreis, 1990). As seen in Figure 6.11, nocodazole treatment induced a notable change in the staining pattern of Flag/GPI-PLD, suggesting that GPI-PLD is present in the Golgi complex. As already indicated in previous experiments, staining for markers such as p23 and sialotransferase (Rojo et al., 1997; Rabouille et al., 1995) and analysis of the dispersion patterns and co-localisation of these molecules with GPI-PLD could be helpful to indicate the precise compartment where GPI-PLD is located within the Golgi complex.

![Non-stimulated and Nocodazole treated cells](image)

**Figure 6.11.** GPI-PLD staining pattern is modified by nocodazole treatment. BHK cells were transiently transfected with the pCMV8-3XFlag/GPI-PLD plasmid. 48 h after transfection, culture medium was changed for fresh medium only or medium containing 20 μM nocodazole. Culture medium of control non-stimulated transfected cells, contained the same amount of DMSO (1%) used to dissolve nocodazole in the stimulated cells (A). After 4h at 37°C, cells were fixed and permeabilised with Triton X-100. Staining was done by using a primary mouse anti-Flag antibody and a secondary FITC-conjugated rabbit anti-mouse antibody.
6.3 DISCUSSION

Despite the potential regulatory roles of GPI-PLD, the actual intracellular site where it is localised is not known. This is an important question to be answered in order to understand the activity and mechanisms that regulate the function of this enzyme.

In this work, transfection experiments of different cell lines using plasmid constructs coding for GPI-PLD fusion protein were done. COS-7, 293T and BHK transfected cells showed that GPI-PLD is expressed mainly in the perinuclear region. These results are in agreement with those obtained when mouse monocyte-macrophages and human keratinocytes stained with antibodies against bovine GPI-PLD showed a bright staining clustered in a perinuclear region (Xie et al., 1993). In some myeloid cells, however, it was demonstrated that the staining for GPI-PLD showed a punctate pattern (Xie and Low, 1994), with aggregates not seen in the cells transfected with plasmids coding for GPI-PLD/GFP or Flag/GPI-PLD fusion proteins. As the three cell lines used in this study, COS-7, 293T and BHK are fibroblast-like cells, it is possible that the punctate staining pattern in myeloid cells is specific for these particular cell types. As it has been reported that GPI-PLD could be taken up from the medium by cultured cells and remain stable inside the cells (Hari et al., 1997), it is possible that GPI-PLD detected in these studies does not represent the endogenous GPI-PLD but that internalised by the cells and therefore is localised in a different subcellular site.

One advantage of the transfection strategy with GPI-PLD fusion proteins used in this work is that GFP or Flag tags are directly linked to GPI-PLD, allowing a direct intracellular localisation of endogenous GPI-PLD. Based on this, it can be considered that the staining pattern seen in COS-7, 293T and BHK cells corresponds to the actual GPI-PLD localisation.

In studies characterising the intracellular distribution of GPI-PLD, Hari et al., measured the GPI-hydrolysing activity in membrane fractions from rat liver. The GPI-PLD activity
was found to be highly enriched in a lysosomal fraction and showed a similar intracellular distribution to that of lysosomal enzymes. These results and the observation that GPI-PLD is taken up by cells led to suggest that once internalised, GPI-PLD is directed to lysosomes (Hari et al., 1996). Because its relative low pH optimum and stability towards proteases, lysosomes would be the intracellular site where GPI-PLD may encounter and cleave GPI-anchored substrates (Hoener et al., 1994; Heller et al., 1994; Li et al., 1994).

Other experiments, however, suggest that GPI-PLD is not located in lysosomes. It has been shown that co-transfection of GPI-PLD and GPI-anchored proteins results in the release of soluble forms of the GPI-anchored proteins. Based on these results, it is believed that intracellular compartments in the secretory pathway may be the site where GPI-PLD is localised (Scallon et al., 1991; Bernasconi et al., 1996). These membrane domains, probably in contrast to the cell membrane, represent a better environment for GPI-PLD action, with cleavage occurring on GPI-anchored proteins in transit to the cell surface (Brown and Rose, 1992).

Studies with streptolysin-O (SLO) support the idea of GPI-PLD localised within intracellular vesicles. It has been demonstrated that incubation of myeloid cells with SLO, which induces pore formation specifically in the plasma membrane, resulted in the release of the cytosolic enzyme lactate dehydrogenase into the medium, whereas GPI-PLD remained cell-associated. Further fractionation of the SLO-permeabilised cells indicated that GPI-PLD was localised mainly in vesicles, endoplasmic reticulum and the Golgi complex (Gravotta et al., 1990).

Analysis of newly expressed GPI-PLD proved that this enzyme does not localise to the cell membrane (Figure 6.3) nor is it associated with caveolae structures (Figure 6.4 and 6.8). However, a certain amount of the expressed GPI-PLD fusion protein co-localises with the dominant-negative mutant form Cav-3 DGV (Figure 6.4). Characterisation of
the cell compartment where Cav-3 DGV is localised indicates that this site is a distinct organelle related to the Golgi-complex and accessed by membrane-bound probes but not by fluid phase markers (Wilson and Colton, 1997). This compartment does not contain EEA1 (an early endosomal marker), lysosomal glycoprotein or cathepsin D, (late endosome/lysosome markers); but it does contain cholesterol. These results thus suggest that this compartment constitutes a novel cholesterol-containing organelle (Roy et al., 1999). The suggestion that GPI-PLD is localised in these cholesterol-containing, Golgi-related compartment is supported by the dispersion in the GPI-PLD fluorescence pattern seen in transfected cells treated with MβCD (a chemical agent that depletes the cell membrane cholesterol) or nocodazole (the chemical that disrupts the structure of Golgi complex) (Figures 6.7 and 6.10). However, the complete characterisation of the intracellular domain where GPI-PLD is localised by using the confocal microscopy approach will be completed only with the inclusion of control experiments using antibodies directed to molecules considered as organelle-specific markers.

Cell fractionation over sucrose density gradients showed that only a small fraction of the expressed GPI-PLD is associated with membranes enriched in Cav-3. Most of the expressed GPI-PLD migrated with the more dense fractions of the sucrose density gradients where cytoplasmic proteins and compartment-specific markers for endoplasmic reticulum, Golgi complex, lysosomes, and mitochondria are also present (Lisanti et al., 1994). The presence of GPI-PLD in the bottom fractions of the density gradients would indicate that a major fraction of the expressed protein is present in vesicles where it is being processed to lower molecular weight forms.

The differences in the molecular weight of immunoreactive GPI-PLD-derived bands observed in the different fractions of the sucrose density gradients were remarkable. These results suggest that GPI-PLD integrity is important to determine its destination. Together with Cav-3 in fractions number 4 of the density gradients, only one GPI-PLD
band of high molecular weight was found (Figure 6.5). Based on this observation, it could be argued that only fully glycosylated is able to co-localise with caveolin in Golgi-related compartment.

The results presented in this chapter demonstrate that GPI-PLD does not co-localise with caveolin within the membrane caveolae structures. Just a fraction of the expressed GPI-PLD co-localises with a dominant-negative mutant form (Cav-3 DGV) within an intracellular compartment. Whether the presence of GPI-PLD within this compartment is important to regulate its activity is not known. What seems to be an important requirement for this association is that GPI-PLD must be fully glycosylated and not proteolytically degraded.
CHAPTER 7  
GENERAL DISCUSSION

7.1 MOUSE LIVER GPI-PLD DNA SEQUENCE

Although the biological function of GPI-PLD has yet to be defined unequivocally, this is the only enzyme described so far with an exclusive specificity for cleavage of GPI anchors (Metz et al., 1994). GPI-PLD has therefore been considered as an important regulator of the release of soluble GPI-anchored proteins from the cell surface (Low and Prasad, 1988). Such cleavage of GPI-anchored proteins produces also inositol phosphoglycans, IPGs, that function as second messengers in the signal transduction of a number of growth factors, including insulin (Rademacher et al., 1994; Jones et al., 1997, Jones and Varela-Nieto, 1998). Thus the potential role of GPI-PLD in the regulation of different cellular functions makes a more extensive characterisation of this enzyme desirable.

One of the first approaches to understand the biological functions of GPI-PLD is the cloning and characterisation of the gene coding for this enzyme. Two different human GPI-PLD cDNAs have been isolated from a liver and a pancreas library (Tsang et al., 1992). Although these two clones showed extensive sequence homology, there were subtle differences suggesting that the human genome may contain two (or more) GPI-PLD genes coding for different amino acid sequences (Tsang et al., 1992). Schofield and Rademacher, however, have recently characterised a human GPI-PLD gene from a set of overlapping clones isolated from a human PAC genome library. In this study clear evidence was given indicating that the human genome only contains a single GPI-PLD gene spanning at least 80 kb (Schofield and Rademacher, 2000).

The differences between pancreas and liver GPI-PLD cDNAs could therefore reflect genetic polymorphisms between the individuals from whom the libraries were made.
This suggestion is possible because most of the differences involve single bases scattered throughout the sequence. Alternatively, the differences might reflect actual sequencing errors.

One aim of this thesis was to characterise the GPI-PLD gene(s) in the mouse. Screening of a mouse liver cDNA library identified one full length cDNA clone with extensive sequence homology to a GPI-PLD cDNA isolated from a mouse pancreatic cell line (LeBoeuf et al., 1998), which was published shortly after my PhD project was started. Despite the high homology between the mouse liver- and pancreas-derived sequences, some differences were found. At that time, these results strongly supported the hypothesis of two different genes encoding for mouse GPI-PLD.

The liver and islet form of mouse GPI-PLD differed in: 9 single bases that changed the amino acid sequence in 4 codons; one insertion of twelve nucleotides (452-464) in the 5' end, and one of six bases in the middle part (1352-1358) of the liver sequence. One deletion of three nucleotides in the liver consensus sequence (1050-1052) and three separate single-nucleotide insertions in the 3' end of the consensus sequence (Figure 3.3) were also detected. In addition, 227 nucleotides in the untranslated 5' end, and 24 nucleotides in the untranslated 3' end of the pancreatic sequence were not present in the liver sequence. If as it is now known for humans, mouse GPI-PLD is encoded by only one gene with multiple small exons, the differences between the two mouse clones could be explained by the expression of alternately spliced GPI-PLD isoforms. Indeed, the insertion of 12 bp in the liver sequence (at nucleotides 452-464) contains the first 5 bp (ATTGA) of the 3' splice acceptor sequence in exon 6 of the human GPI-PLD genomic sequence (Schofield and Rademacher, 2000).

The related phospholipase D1 (PLD1) that cleaves phosphatidylcholine, does exist in alternative spliced forms. One important consequence of this is that with the same enzymatic activity, the different isoforms of PLD1 can be located in separate
intracellular compartments to receive and/or respond to different kinds of extracellular signals (Hammond et al., 1997). Because of the potential regulatory roles of GPI-PLD, the tissue-specific expression of alternately spliced forms could be important to explain its intracellular localisation or the regulation of its function.

RPAs carried out in this thesis using antisense probes specifically designed to detect putative alternately spliced GPI-PLD isoforms identified only one single transcript with sequence identity to the liver GPI-PLD in all the tissues where GPI-PLD expression could be detected (Chapter 3). These results were substantiated by RT-PCR amplification followed by sequencing of the generated GPI-PLD fragments. Furthermore, Southern blot analysis of mouse genomic liver DNA showed no evidences that more than one gene coding for mouse GPI-PLD were obtained (Figure 3.8). Collectively these data strongly suggest that mouse GPI-PLD, as in humans, is encoded by only one single gene with a sequence identical to that determined for the liver cDNA clone in this thesis, which appears not to be regulated by alternative splicing.

7.2 MOUSE GPI-PLD EXPRESSION.

Studies on the functional role of GPI-PLD have been limited partly because of a general lack of information regarding its sites of synthesis and release into the circulation. A second aim of this thesis was to analyse the expression of mouse GPI-PLD. This was achieved by using a combination of Northern blot analysis, RPAs and RT-PCR using RNA isolated from different tissues. The results showed a single transcript of ~4 kb in liver, antrum, salivary glands and lung, and one transcript with a higher molecular weight in brain RNA (Figure 3.9).

As observed for the mouse (Figure 3.9), the rat GPI-PLD transcripts expressed in liver and brain differs slightly in size (Schofield et al., 2002). As transcripts from liver and
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brain seem to have the same translatable sequences, as judged by the RPAs (Figures 3.13-3.15), the size differences could either be due to sequence differences in the 3' non-translated region or alternatively differences in the 5' end, implying two tissue-specific promoters. It would be interesting to analyse these regions and try to find out if there are any sequences, which could affect the RNA stability and hence, the GPI-PLD activity. Another important point is that the different relative amounts of mRNA expressed in brain and liver suggest that tissue- or cell-specific mechanisms regulating the expression of GPI-PLD might exist.

Liver is the major tissue producing GPI-PLD as I observed here and consistent with the results obtained by others (LeBoeuf et al., 1998; Deeg et al., 2001b). The expression analysis described in this thesis was extended by analysing the expressed sequence tags (EST) database from the NCBI. Although this analysis showed that about 30% of the samples are derived from liver, it is clear that GPI-PLD is widely expressed in many different tissues, albeit at very low levels.

7.3 REGULATION OF GPI-PLD EXPRESSION.

The mechanisms responsible for the regulation of GPI-PLD expression have not been elucidated yet. A third aim of this thesis was to establish whether the genetic background of different mouse strains affects the expression levels of GPI-PLD. Previous studies of serum GPI-PLD activity showed significant variation amongst different inbred mouse strains tested. These results implied that genetic factors are important in the control of GPI-PLD levels (LeBoeuf et al., 1998). Having established that liver is the major source of GPI-PLD in mouse, the expression of GPL-PLD in this organ was evaluated in different mouse strains. RPAs showed variations in the GPI-PLD RNA level when different mouse strains were compared (CBA/Ca>C56BL/10>BALB/c>DBA/1=DBA/2>NZW, Figure 3.16 A and B). In other words, the levels of GPI-PLD mRNA observed in normal strains were relatively higher compared
to mouse strains prone to develop autoimmune diseases such as systemic lupus erythematosus or arthritis.

More evidence of the importance of genetic factors in the regulation of GPI-PLD expression were obtained from the analysis of liver GPI-PLD mRNA expression level in different models of diabetes. In this thesis it was found that nonobese diabetic (NOD) mice, a spontaneous model for type 1 diabetes, show an increased level of GPI-PLD mRNA expression when they became diabetic (Figure 3.17 A and B). In contrast, the ob/ob and db/db mice appeared to have lower levels of GPI-PLD mRNA expression compared to CBA/Ca mice although I did not use the most appropriate control mouse strain (C57BL/6) for comparison (Figure 3.18 C and D). These two strains are animal models of type 2 diabetes. Although hyperglycaemia is a characteristic clinical manifestation in both diabetes types, the pathogenesis of the diseases is quite different. Type 1 diabetes is an autoimmune disease characterised by the selective destruction of the insulin-producing cells in the pancreas resulting in insulin deficiency (Bottazzo et al., 1974; MacCuish et al., 1974). Type 2 diabetes is characterised by the lack of insulin responsiveness and is often associated with obesity (Shafrir, 1993). The pathology in the ob/ob and the db/db mice is caused either by the functional deletion of the leptin growth factor (in the ob/ob strain), or the long transmembrane form of the leptin receptor (in the db/db mouse) (Coleman, 1974).

In this context it is interesting to note that GPI-PLD has been mapped to regions that both in humans and NOD mice contain susceptibility genes for type 1 diabetes. The human GPI-PLD gene maps to the short arm of human chromosome 6 (Schofield and Rademacher, 2000) just telomeric for the major histocompatibility complex. This region contains the marker D6S2223 that has been associated with susceptibility to type 1 diabetes (Lie, et al., 1999a, b). The mouse GPI-PLD gene maps to mouse chromosome 13 (LeBoeuf et al., 1998) in a region containing the diabetes susceptibility locus idd14 (McAleer et al., 1995) and with extensive homology to the segment of
human chromosome 6 containing the GPI-PLD gene and D6S2223 (NCBI Human-
mouse genome Map). These results suggest then that at least in the liver as yet
unknown genetic factors regulate GPI-PLD expression.

The results presented in this thesis and in recent studies (Bowen et al., 2001; Deeg et
al., 2001b) suggest that insulin and/or glucose may affect the liver GPI-PLD mRNA
expression. In order to find more evidence to support this idea, the liver GPI-PLD
mRNA expression in a different animal model of diabetes was evaluated in this thesis.
This analysis showed that streptozotocin (STZ)-induced diabetic mice have higher
levels of GPI-PLD mRNA when compared to untreated control animals (Figure 3.18A).
In addition, one study with STZ-induced diabetic rats showed that administration of
insulin decreased the high levels of serum GPI-PLD and mRNA in the liver of these
animals (Schofield et al., 2002). On the other hand, it has been recently shown that
insulin stimulates the expression of GPI-PLD by pancreatic cells in ob/ob mice (Bowen
et al., 2001). All these results suggest that insulin and/or glucose may regulate the
expression of GPI-PLD in a tissue-specific manner. In addition to this, one interesting
result was the relatively higher level of GPI-PLD RNA expression detected in pre­
puberty NOD mice compared to post-puberty, which suggests a possible role of
hormonal factors regulating GPI-PLD expression (Figure 3.17).

In a different disease model, GPI-PLD has also been implicated in atherosclerosis.
Macrophages present in atherosclerotic lesions of coronary arteries produce an
elevated level of GPI-PLD, suggesting a role for the enzyme in the pathogenesis, and a
possible link between GPI-PLD and the oxidative stress is suggested by the
observation that exposure of macrophages to H₂O₂ results in an increase of GPI-PLD
RNA expression (O'Brien et al., 1999). This may not be a direct consequence of H₂O₂
because recent observations using mouse macrophage cell lines showed that H₂O₂
decreases the level of GPI-PLD expression (Du et al., 2001).
7.4. MOUSE LIVER GPI-PLD INTRACELLULAR LOCALISATION AND ACTIVITY.

Despite the potential regulatory roles of GPI-PLD, little is known about the actual intracellular compartment where it is localised and exerts its biological function. Different studies have demonstrated that serum or purified GPI-PLD catalyses the hydrolysis of GPI-anchored proteins only in the presence of detergents or other agents that may induce conformational changes in the enzyme (Davitz, 1989; Low and Huang, 1991; Bergman et al., 1994). *In vivo,* the activity of GPI-PLD has been observed when both GPI-PLD and GPI-anchored proteins have been expressed simultaneously (Scallon et al., 1991; Bernasconi et al., 1996; Kung et al., 1997). These results may suggest that: 1) GPI-PLD acts only on particular cell types or in concert with other cofactors, 2) it is secreted in a transiently active state close to its substrates, 3) it is restricted to an intracellular location in which for some unknown reasons, GPI-anchors are especially sensitive to its action, or 4) that it is transported from the extracellular medium to particular subcellular compartment(s) where it becomes activated so it can exert its biological function (Davitz, 1989; Low and Huang, 1991; Bergman et al., 1994).

In the absence of GPI-PLD specific monoclonal antibodies, a set of plasmids encoding mouse GPI-PLD fused with different epitope tags were constructed and expressed in different cell lines with the aim of further analyse the intracellular location and activity of *de novo* synthesised GPI-PLD.

Previous studies localised GPI-PLD to intracellular compartments highly enriched in lysosomal fractions with a similar intracellular distribution to that of lysosomal enzymes (Hari et al., 1996). These results suggest that GPI-PLD is internalised from the extracellular medium and is directed to lysosomes where it encounters and cleaves GPI-anchored substrates after proper activation (Hari et al., 1997). Other studies,
however, have localised GPI-PLD within endoplasmic reticulum (ER)- and Golgi complex-related vesicles (Xie and Low, 1994). Such an intracellular distribution was also suggested in this thesis by intracellular staining and confocal microscopy analysis of transfected cells with GPI-PLD-encoding plasmids (Figure 6.2). The discrepancy between these observations may be explained considering that the lysosome-associated GPI-PLD may be derived from circulating serum GPI-PLD, whereas the ER/Golgi-associated enzyme is synthesised de novo.

GPI-anchored proteins, the potential substrates of GPI-PLD, are localised on the cell surface often associated with lipid rafts or caveolae which are enriched in signal transduction complexes as discussed in the introduction (Parton and Simons, 1995). Signalling molecules associated with/regulated by caveolin, the major structural component of caveolae (Schlegel et al., 2001), contain the sequence motif \( \Psi X X X X \Psi X X \Psi \), where \( \Psi \) represents an aromatic residue and \( X \) any other amino acid (Couet et al., 1997a,b).

Mouse GPI-PLD contains the sequence FLVEQFQDY, which fits with the caveolin-binding motif, suggesting that GPI-PLD could interact with caveolin in the caveolae. Supporting this, PLD2 one mammalian isoform of PLD has been found enriched in caveolin-rich membrane domains (Czarny et al., 1999). However, because of the topology of caveolin and GPI-PLD, an interaction between these two molecules is difficult to be established. Being GPI-PLD a secreted protein its presence in the extracellular milieu would not allow the interaction with caveolin, which is associated with the inner leaflet of the membrane (Figure 7.1). In addition, caveolin-associated proteins are lipid-modified. The lack of this protein-modification in GPI-PLD would exclude a direct interaction between these two molecules.

The results obtained in this thesis corroborated that neither Flag/GPI-PLD nor GPI-PLD/GFP fusion proteins are associated or located in the cell membrane of transfected
CHAPTER 7. GENERAL DISCUSSION.

cells (Figures 6.3, 6.9 and 6.10). Cells co-transfected with plasmids coding for GPI-PLD and caveolin-3 (Cav-3) showed furthermore that GPI-PLD is not associated with Cav-3 nor co-localise in caveolae at the cell membrane (Figures 6.5 and 6.6). The expressed GPI-PLD/GFP fusion protein, however, was localised in the same compartment as a dominant-negative mutant form of Cav-3 (Cav-3 DGV) (Figure 6.6), which is a novel cholesterol-rich organelle associated with the cis-Golgi (Wilson and Colton, 1997; Roy et al., 1999). The presence of GPI-PLD in this cholesterol-containing compartment is also suggested by the dispersion in the GPI-PLD/GFP fluorescence pattern when transfected cells are treated with methyl-β-cyclo dextrin (MβCD) or nocodazole.

Co-localisation was also seen following cell fractionation over sucrose gradients, although only a small fraction of the expressed and fully glycosylated GPI-PLD was actually present in the Cav-3 DGV-enriched fractions (Figure 6.7). Most of the cell-associated GPI-PLD sedimented at more dense fractions of the gradient, where ER and cytosolic proteins also fractionate. These fractions contain both fully glycosylated and partly degraded GPI-PLD polypeptide. The apparently contradictory results obtained by the subcellular fractionation and confocal microscopy may be reconciled by considering that GPI-PLD, which has no membrane attachment, is located in the lumen of these Cav-3 DGV-enriched compartments.

Other secreted or cell surface associated proteins – as exemplified by Cav-3 in the present experiments – show very little degraded protein or protein that could be regarded as ER-associated when compared with that of GPI-PLD. The presence of GPI-PLD in high density fractions of the sucrose gradient could be also explained by the tendency of GPI-PLD to form aggregates in the absence of detergents (Deeg et al., 2001a). I used a prokaryotic expression system to synthesise the three GPI-PLD domains which can be generated following trypsin digestion of GPI-PLD purified from
bovine serum (Li et al., 1994). However, each of the domains was insoluble in absence of high concentrations (>6M) of urea (Lund and Flores, unpublished data). This was also seen with bovine GPI-PLD fragments purified after trypsin treatment (Heller et al., 1994). Because of its highly hydrophobic nature, newly synthesised GPI-PLD may be retained in ER in association with chaperones until its partner or carrier protein is produced. The association of GPI-PLD with its carrier protein would release GPI-PLD from the chaperone allowing its transport along the secretory pathway (Figure 7.1) in a similar way to that described for the synthesis and assembly of the major histocompatibility complex class II molecules (Anderson and Kresswell, 1994).

ApoA-I could represent the partner GPI-PLD requires to be transported along the secretory pathway. In liver, where most of the synthesis of apolipoproteins (Shepherd, 1994) and GPI-PLD occur, association between apoA-I and GPI-PLD could explain why most of the serum GPI-PLD is associated with apo-AI (Deeg et al., 2001a). In other GPI-PLD-expressing cells, the carrier protein may be different or expressed in much less quantity, which may explain the intracellular retention, and extensive degradation of GPI-PLD observed in this thesis (Figure 4.7). It would be interesting to conduct transfection experiments of GPI-PLD in hepatic cells as natural sources of apoA-I or using plasmids coding for this molecule in kidney-derived cell lines (as those used in this thesis) and then evaluate the intracellular localisation, secretion and activity of GPI-PLD.

In relation to GPI-PLD activity, the results obtained in this thesis were consistent with previous reports (Scallon et al., 1991; Bernasconi et al., 1996; Kung et al., 1997). Here was demonstrated that endogenous GPI-PLD can cleave GPI-anchored proteins, which are synthesised simultaneously with GPI-PLD. Co-transfection with plasmids encoding PLAP and GPI-PLD produced soluble PLAP, with the cleavage being specifically catalysed by GPI-PLD because co-expression of a GPI-PLD antisense fragment inhibited the release of PLAP (Figure 5.4). Supporting this finding, Wilhelm et
had previously demonstrated that the expression of a GPI-PLD antisense mRNA molecule, inhibited the active release of the receptor for urokinase plasminogen activator (CD87) in human ovarian cancer cells (Wilhelm et al., 1999). All these results indicate that intracellular compartments along the secretory pathway may be the sites where GPI-PLD activity takes place. It is possible that intracellular membrane domains, probably different to the cell membrane represent a better environment for GPI-PLD action, with cleavage occurring on GPI-anchored proteins in transit to the cell surface (Brown and Rose, 1992).

The different sites for phosphorylation found in the GPI-PLD sequence (Table 3.1) suggest a high regulation of its enzymatic activity. The reduction in GPI-PLD activity observed after phorbol-12-myristate-13 acetate (PMA) stimulation of GPI-PLD/PLAP co-transfected cells (Figure 5.5) indicates that GPI-PLD activity can be regulated by protein kinase C (PKC). In a previous study, it was reported that PMA stimulation of myeloid cells resulted in an increase of GPI-PLD secretion (Xie and Low, 1994), and this is probably related to a decrease in its activity.

Figure 7.1 summarises a model to explain the function of GPI-PLD based on the information generated in this thesis and that available in the literature. Because of its hydrophobic nature, de novo synthesised GPI-PLD could be retained in the ER (1), probably in association with chaperon-like proteins, until it associates with a carrier in order to be transported along the secretory pathway. Some evidence suggests that whilst in ER or during its transport to the exterior, GPI-PLD can exert its catalytic activity (red arrows, in the figure) and cleave GPI anchors (Scallon et al., 1991; Bernasconi et al., 1996). As suggested by the co-localisation with Cav-3 DGV observed in this thesis, GPI-PLD could become associated with cholesterol-rich, Golgi-related compartments (2). Whether the presence of GPI-PLD in this compartment is important for its activity needs further experiments to be evaluated for example by treating GPI-PLD transfected cells with agents that disrupt the Golgi complex.
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GPI-PLD synthesized and secreted from the liver – the main source of GPI-PLD - can be taken up by cells possibly through a caveolae-mediated mechanism (3). The concentration of GPI-anchored proteins in this membrane compartment makes it a potential site for GPI-PLD to exert its activity, as has been reported for PLD1 (Kim et al., 1999). An alternative route followed by secreted GPI-PLD could be suggested from studies on internalisation of GPI-PLD and fractionation of membranes from liver cells. Two different studies have located GPI-PLD in membranes enriched in lysosomal enzymes, and have shown that neuroblastoma cells can internalise GPI-PLD in a concentration- and time-dependent manner (Hari, et al., 1996, 1997). These observations would suggest that extracellular GPI-PLD is internalised and directed to lysosomes (4) where it could find GPI-anchored proteins and cleave them off.

As shown in this thesis caveolin and de novo synthesized GPI-PLD do not interact in the caveolae because they are distributed on opposite sides of the membrane. Despite this, a direct interaction between caveolin and GPI-PLD could be possible with the translocation of caveolin to the luminal space of the ER/Golgi compartments. One study has shown that caveolin redistributes constitutively to the lumen of the ER in response to cholesterol oxidation, as part of a mechanism related with cholesterol transport (Smart et al., 1996). It has been suggested that at some stage in the caveolae internalisation cycle, caveolin-1 can enter the cytoplasm as a soluble protein embedded in a protein/lipid complex (5). Once in the cytoplasm, the soluble caveolin may go to the ER and enter the lumen of this organelle (Uittenbogaard, et al., 1998; Liu et al., 2002) where it is incorporated in high-density lipoprotein particles that are secreted by the cell (Liu, et al., 1999). With this information, it would be interesting to analyse whether the trafficking mechanisms of caveolin inside the cells are important for the activity of GPI-PLD.
Figure 7.1. A model of intracellular localisation and activity for GPI-PLD.
7.5 GPI-PLD in vivo ACTIVITY

The release of CD14, a GPI-anchored protein, by macrophages has been recently described by Delgado et al. In that work it was demonstrated that macrophages simultaneously stimulated with lipopolysaccharide (LPS) and vasoactive intestinal peptide (VIP) – or LPS and PMA – rapidly down-modulated the surface level of CD14 by releasing it to the medium. It was also demonstrated that LPS and VIP stimulation did not affect the CD14 mRNA levels, which suggested the participation of phospholipases in the cleavage of the GPI-anchor of CD14 (Delgado et al., 1999a).

Considering this evidence, the possible role of GPI-PLD in the shedding of CD14 was investigated in this thesis. CD14 is anchored to the cell surface by GPI (Haziot et al., 1988) and function together with toll-like receptor 4 (TLR4) as the receptor complex for LPS (Aderem and Ulevitch, 2000). One possible scenario is that when LPS is encountered, an active receptor complex is formed which locates to the lipid rafts or caveolae where it can interact with G proteins (Solomon et al., 1998). The signals mediated by LPS and those by VIP, a neurotransmitter that also has immunoregulatory properties (Delgado et al., 1998; Delgado et al., 1999b) could result in the activation of GPI-PLD and the subsequent release of CD14.

Since the macrophage cell line RAW267.4 expresses both GPI-PLD (this thesis, Figure 3.15 B; Xie and Low, 1994) and CD14 (Ziegler-Heitbrock et al., 1993), it was selected for the analysis. Unfortunately, I failed to detect any soluble CD14 released into the supernatant following stimulation of the macrophages with VIP and LPS (Figures 5.6 and 5.7). It is possible that this failure was due to the methods I used, which were not sensitive enough to detect low concentrations of soluble CD14. On the other hand, the effect of LPS on the expression of GPI-PLD in macrophages is quite controversial. It has been shown recently that LPS inhibited the expression of GPI-PLD mRNA, whilst at the same time increased the level of CD14 on the cell membrane (Du et al., 2001).
The negative results obtained in these experiments did not allow analysing the role of GPI-PLD in the release of GPI-anchored proteins.

The detection of soluble forms of several GPI-anchored proteins in other systems has been well documented. These studies suggest that the GPI-anchors are cleaved by phospholipases because the soluble forms of GPI-anchored proteins contained ethanolamine, which would have been removed if the cleavage had been mediated by a protease. The soluble forms of GPI-anchored proteins were not secreted because treatment with brefeldin A which inhibits the budding and fusion of transport vesicles between the ER and Golgi complex did not inhibit the GPI-anchored proteins release (Gennarini et al., 1984; Medof et al., 1987; Almqvist and Carlsson, 1988; Klemens et al., 1990; Metz et al., 1994; Nemoto et al., 1996; Lieberheimer et al., 1997; Delgado et al., 1999a; Wilhelm et al., 1999).

Evidence pointing to a cleavage mechanism mediated by GPI-PLD has been obtained. This evidence include the detection of phosphatidic acid as a reaction product, the lack of the cross-reacting determinant (CRD) cryptic epitope in soluble forms of GPI-anchored proteins, and the inhibitory effect of 1,10-phenanthroline (Brunner et al., 1994; Nemoto et al., 1996). In addition, the inhibition of the release of GPI-anchored proteins has been described in cells transfected with a GPI-PLD antisense mRNA (Wilhelm et al., 1999).

There is, however, experimental evidence to suggest that GPI-PLD may not be the only phospholipase involved in the cleavage of GPI-anchors. Recent studies have demonstrated a cleavage mechanism of GPI-anchored proteins mediated by GPI-PLC supporting an early indication of the role of a PLC in the release of 5'-nucleotidase from bovine cerebral cortex and the electric organ of the electric ray (Vogel et al., 1992). Hooper and colleagues have recently shown that the release of membrane dipeptidase from the cell surface of adipocytes and porcine proximal tubules, respectively, might...
have been catalysed by a GPI-PLC. The soluble forms of the GPI-anchored dipeptidases were recognised by anti- CRD antibodies and their release depended on the presence of Ca$^{2+}$, which strongly supports a mechanism mediated by GPI-PLC (Movahedi and Hooper, 1997 Park et al., 2001). Future characterisation of this putative GPI-PLC will determine whether it has a function restricted to cleavage of GPI-anchored proteins or if it has a broader specificity.

Released GPI-anchored proteins may have tasks distinct from their cell-associated function. For instance, recent studies have shown that soluble CD14 regulates the activation and function of human T cell clones and mononuclear cells (Rey-Nores et al., 1999). Soluble CD14 regulates also the proliferation, differentiation and production of specific cytokines and immunoglobulin isotypes by monocyte-like cell lines and B lymphocytes (Labeta et al., 1993; Arias et al., 2000; Filipp et al., 2001). Other solublised GPI-anchored proteins such as heparan sulphate proteoglycan and ADP-ribosyltransferase stimulate the proliferation of bone marrow and stem cells (Brunner et al., 1994), and the cytotoxic activity of T cells, respectively (Wang et al., 1994; Nemoto et al., 1996).

Because GPI-anchored proteins are expressed in many different cell types combined with their diverse biological functions, the mechanisms that control their release might be highly regulated and could conceivably involve the participation of different phospholipases. The accumulated experimental evidence indicating that both GPI-PLD and GPI-PLC can catalyse the cleavage of GPI-anchored proteins suggests that depending of the cell type, either GPI-PLD or GPI-PLC may be active under specific conditions of stimulation. The possibility that at least two different phospholipases control the release of GPI-anchored proteins point also to different intracellular locations where the cleavage occurs. The complete characterisation of the enzymes involved in this process, including their intracellular location, structure and inhibitors and activators, is very important to understand the mechanisms that regulate the
release of GPI-anchored proteins, a chemical process with important biological implications.

7.6 CONCLUSIONS

Taken together, the results obtained in this work indicate that mouse GPI-PLD is encoded by only one gene, which is processed to a single transcript, possibly in a tissue-specific manner. The differences between the pancreas and liver-derived GPI-PLD sequences are the result of sequencing errors in the pancreas sequence.

Liver is the major tissue producing GPI-PLD, although it seems that most cells produce this enzyme at low concentrations. As suggested by analysis of GPI-PLD expression in different mouse strains, genetic factors seem to be important to determine the levels of GPI-PLD RNA expression. In relation to diabetes animal models, changes in the response to and levels of insulin and/or glucose might also be important in the regulation of GPI-PLD expression.

De novo synthesised GPI-PLD does not co-localise with caveolin within caveolae structures at the cell membrane. However a fraction of expressed GPI-PLD co-localises in a cis-Golgi-related compartment rich in cholesterol. Because of the potential regulatory role in different biological mechanisms and the apparent involvement in pathological states, a complete understanding of the biology of GPI-PLD is necessary. Information generated in this thesis related with the regulation of the expression and intracellular localisation (see model in Figure 7.1) can be used as springboards to define further experiments that will be helpful to understand the function of this molecule.
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