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A UDP-glucose deficient mutant cell line as a model to study the cytotoxicity of *Clostridium perfringens* PLC

by

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SUMMARY

A Chinese Hamster fibroblast mutant cell line, deficient in UDP-glucose (UDP-Glc) and hypersensitive to *Clostridium perfringens* phospholipase C (PLC) was used in this study to determine some of the molecular consequences of a cellular UDP-Glc deficiency. Furthermore, using this cell as a model, structure/function studies were performed to identify residues critical for the cytotoxic activity of PLC.

It was found that the reason for the cellular UDP-Glc deficiency is a point mutation in the gene that encodes the UDP-Glc pyrophosphorylase (UDPG:PP), the enzyme that catalyzes UDP-Glc synthesis. The mutation changes the conserved glycine 115 to aspartic acid in the protein product. Protein analysis of cell lysates showed that the mutant cell overproduces seven stress proteins: one mitochondrial chaperone (GRP75) and six chaperones of the endoplasmic reticulum (GRP58, ERp72, GRP78, GRP94, GRP170 and calreticulin). These proteins are also upregulated in cells cultured under hypoxia or glucose starvation as well as in ischemic tissues.

To clarify whether there is a connection between the UDP-Glc deficiency and the overproduction of stress proteins and the hypersensitivity to the PLC, stable transfectant cells were prepared using a wild type UDPG:PP cDNA. Transfectant clones increased their UDP-Glc concentration and produce normal amounts of calreticulin and the GRPs, indicating that the UDP-Glc deficiency induces their overproduction. Exposure of the transfectant clones to PLC demonstrated that a cellular UDP-Glc deficiency causes hypersensitivity to the cytotoxic effect of this phospholipase.

The UDP-Glc deficient cell was used to characterize the structural determinants responsible for the cytotoxic activity of PLC. Experiments with genetically engineered PLC variants showed that the sphingomyelinase activity and the C-terminal domain are required for its cytotoxic effect. In addition, *in vivo* experiments demonstrated that the sphingomyelinase activity and the C-terminal domain are also needed for myotoxicity. The toxic activities of PLC variants harboring single amino acid substitutions in aspartic acid residues, which bind calcium, and tyrosine residues of the putative membrane-interacting region at the C-terminal domain were studied. These residues were found to be critical for the hemolytic, cytotoxic and myotoxic activities of PLC.

Since UDP-Glc is required for the synthesis of membrane glycoconjugates, their role in the sensitivity to PLC was studied. It was found that inhibition of glycoproteins or proteoglycans synthesis/processing does not affect the sensitivity to PLC, whereas inhibition of glycosphingolipid synthesis sensitizes cells to this toxin. Furthermore it was demonstrated that complex gangliosides protect hypersensitive cells from the cytotoxic activity of PLC and prevent the membrane disrupting effect of this toxin in artificial membranes.

In conclusion, this work revealed that a cellular UDP-Glc deficiency induces the upregulation of a set of stress proteins important for cell survival under ischemic like conditions and furthermore provide new insights to understand the molecular mechanism of action of *C. perfringens* PLC.

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Flores-Díaz, M**., Alape-Girón, A., Persson, B., Pollesello, P., Moos, M., Eichel-Streiber, Cv., Thelestam, M., and Florin, I. 1997. Cellular UDP-glucose deficiency caused by a single point mutation in the UDP-glucose pyrophosphorylase gene. *J. Biol. Chem*. 272, 23784-23791.

II**. Flores-Díaz, M**., Alape-Girón, A., Florin, I., Higuita, J. C., Pollesello, P., Okada, T., Moos, M., Cordula, C., Bergman, T., Eichel-Streiber, Cv., Mori, K., and Thelestam, M.. 2001. A cellular UDP-glucose deficiency causes overproduction of stress proteins in the mitochondria and the endoplasmic reticulum (manuscript).

III**. Flores-Díaz, M**., Alape-Girón, A., Titball, R. W., Moos, M., Guillouard, I., Cole, S., Howells, A. M., Eichel-Streiber, Cv., Florin, I. and Thelestam, M. 1998. UDP-glucose deficiency causes hypersensitivity to the cytotoxic effect of *Clostridium perfringens* phospholipase C. *J. Biol. Chem*. 273, 24433-24438.

IV. Alape-Girón, A*., **Flores-Díaz, M.***, Guillouard, I., Naylor, C. E., Titball, R. W., Rucavado, A., Lomonte, B., Basak, A. K., Gutiérrez, J. M., Cole, S. and Thelestam, M. 2000. Identification of residues critical for toxicity in *Clostridium perfringens* phospholipase C, the key toxin in gas gangrene. *Eur. J. Biochem*. 267, 5191-5197.

V. **Flores-Díaz, M.**, Alape-Girón, A., Clark, G., Ichikawa, S., Hirabayashi, Y., Gutierrez, J. M., Titball, R. W., and Thelestam, M. 2001. Cellular deficiency of gangliosides causes hypersensitivity to *Clostridium perfringens* phospholipase C. *J. Biol. Chem*. (submitted).

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*The authors contributed equally to this work

ABBREVIATIONS

CHO	Chinese Hamster ovary cells
C-terminal	carboxy terminal
DAG	diacylglycerol
DL-PPMP	DL-threo-1-phenyl palmitoylamino-3-morpholino-1-propanol
Don Q	Chinese Hamster mutant cell line
Don QR	Chinese Hamster revertant cell line
ER	endoplasmic reticulum
GalCer	galactosylceramide
Glc-1-P	glucose-1-phosphate
Glc-6-P	glucose-6-phosphate
GlcCer	glucosylceramide
GM-95	Mouse mutant melanome cells
GRPs/ORPs	glucose/oxygen regulated proteins
GSL(s)	glycosphingolipid(s)
IP3	inositol-3-phosphate
LDH	lactate dehydrogenase
N-terminal	amino terminal
PdtCho	phosphatidylcholine
PKC	protein kinase C
CK	creatine kinase
PLC	phospholipase C
SiaAc	sialic acid
SM	sphingomyelin
UDPG:CGT	UDP-glucose:ceramide glucosyltransferase
UDPG:PP	Uridine diphospho-glucose pyrophosphorylase
UDP-Gal	Uridine diphospho-galactose
UDP-Glc	Uridine diphospho-glucose
UDP-GlcUA	Uridine diphospho glucuronic acid
UPR	Unfolded Protein Response

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INTRODUCTION

In this work a Chinese Hamster mutant cell line (Don Q), deficient in UDP-Glc and hypersensitive to *Clostridium perfringens* phospholipase C (PLC), was used as a tool to study the cellular consequences of a low UDP-Glc level. In the first part of the study the reason for the low UDP-Glc level was found and some of the consequences of the cellular UDP-Glc deficiency determined. In the second part of the work, the reason for the cellular hypersensitivity to *C. perfringens* PLC was clarified and, in addition, the cell line was used in structure-function studies which allowed to identify some of the residues critical for its cytotoxic activity.

UDP-glucose: synthesis and metabolism

The synthesis of UDP-Glc occurs in the cytoplasm and depends on the amounts of glucose-6-phosphate (Glc-6-P) and UTP available in the cell. Two enzymes participate in the pathway of UDP-Glc synthesis: phosphoglucomutase, which interconverts Glc-6-P and glucose-1-phosphate (Glc-1-P); and UDP glucose pyrophosphorylase (UDPG:PP), which catalyzes the formation of UDP-Glc from UTP and Glc-1-P (Fig. 1).

UDP-Glc is used as a precursor in the synthesis of glycogen (1) and it is required for the entry of galactose into glycolysis (2). Furthermore it is necessary for the synthesis of UDP-galactose (UDP-Gal) and UDP-glucuronic acid (UDP-GlcUA) (3), two main sugar donors in the synthesis of glycoconjugates. UDP-Glc is also an essential glucose donor during the synthesis of cell surface glycoproteins (4), and glycosphingolipids (GSLs) (5). This metabolite is actively transported to the lumen of the endoplasmic reticulum (ER) (6) where it is used by the UDP-Glc:glycoprotein glucosyltransferase during the quality control of newly synthesized glycoproteins (4). The glycosylation of N-linked glycoproteins occurs in the ER, where a core oligosaccharide is transferred *in block* to specific asparagine residues of the nascent

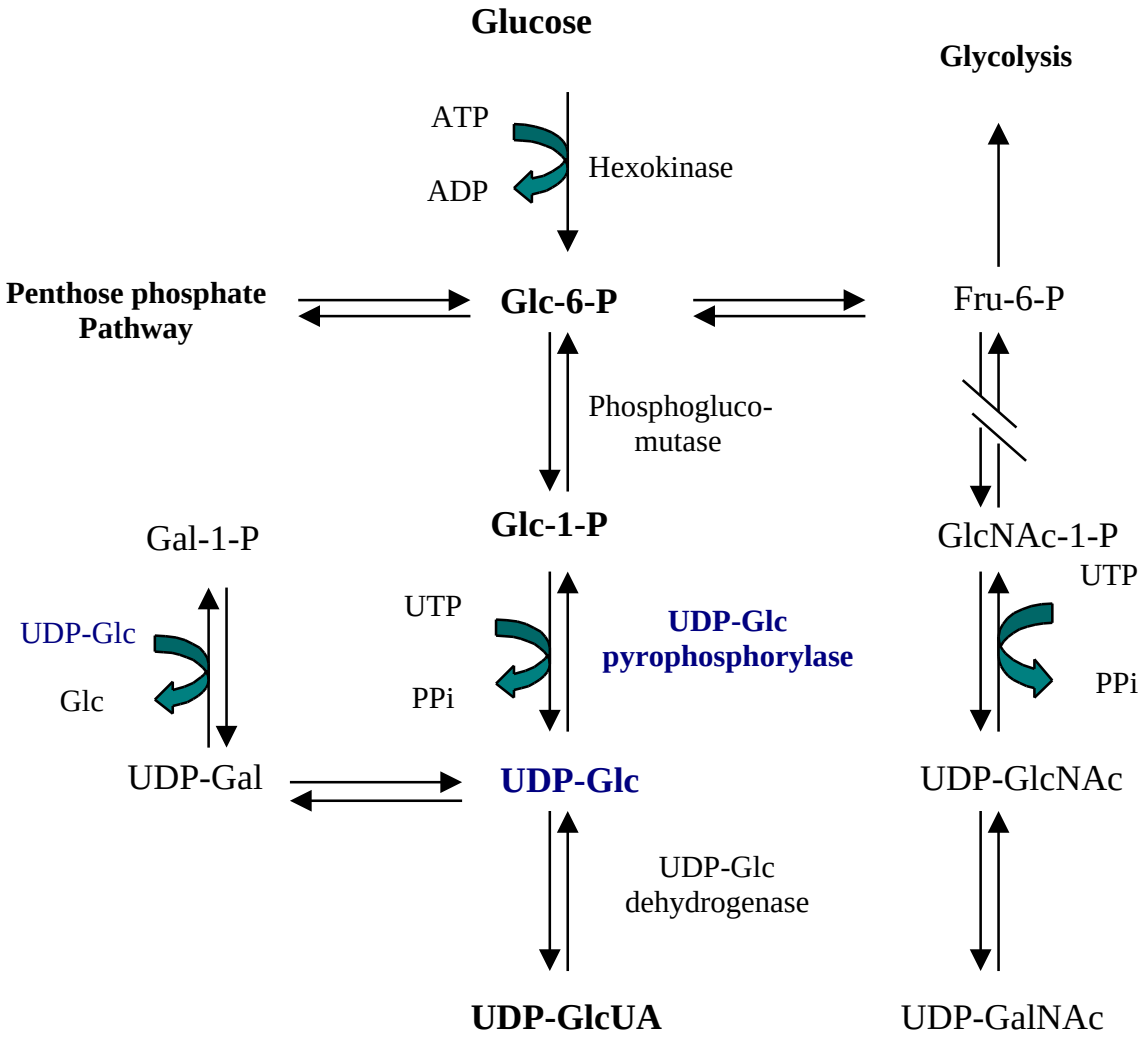

Fig. 1 Pathway of synthesis of UDP-hexoses

polypeptide chains. The synthesis of this oligosaccharide structure occurs on dolichol at the ER membrane and involves the stepwise addition of monosaccharide units from activated sugars, including three glucoses donated by UDP-Glc (4). Glycoproteins are then subjected to extensive modification as they mature and move through the ER and via the Golgi complex to their final destinations (4).

The synthesis of glycosaminoglycans, the polysaccharide chain of proteoglycans occurs in the lumen of the Golgi apparatus (3). It employs sequential addition of monosaccharides rather than a *bloc* transfer of pre-assembled oligosaccharide. The process is initiated by sequential addition of four monosaccharides (xylose, two galactoses and a glucuronic acid) to a serine or a threonine residue of the proteoglycan's protein portion (3). Thus, the synthesis of this linker tetrasaccharide requires UDP-Gal and UDP-GlcUA as precursors (3).

GSLs are composed of a variable carbohydrate moiety linked to ceramide, and derived either from galactosylceramide (GalCer) or glucosylceramide (GlcCer) (5). GalCer is synthesized from ceramide and UDP-Gal in the lumen of the ER, whereas GlcCer is synthesized from ceramide and UDP-Glc on the cytosolic face of the Golgi apparatus membrane (5). Ceramide, a common precursor of GSLs and sphingomyelin (SM), is either synthesized *de novo* from L-serine and palmitoyl CoA or by a salvage pathway from sphingosine (Fig. 2). In mammalian cells the main derivatives of GlcCer are gangliosides, which depending on the number of sialic acid (SiaAc) moieties and the sites where they are attached are grouped in the series o, a, b, or c (6). In addition to *de novo* synthesis, GSLs are selectively internalized via a clathrin-independent pathway and recycled from the endosomal pathway to the Golgi (7).

A UDP-Glc deficiency has been reported to occur in cells cultured under low glucose concentration or hypoxia (8, 9), as well as in insulin-dependent tissues of diabetic organisms (10). However the molecular consequences of this deficiency have not been established.

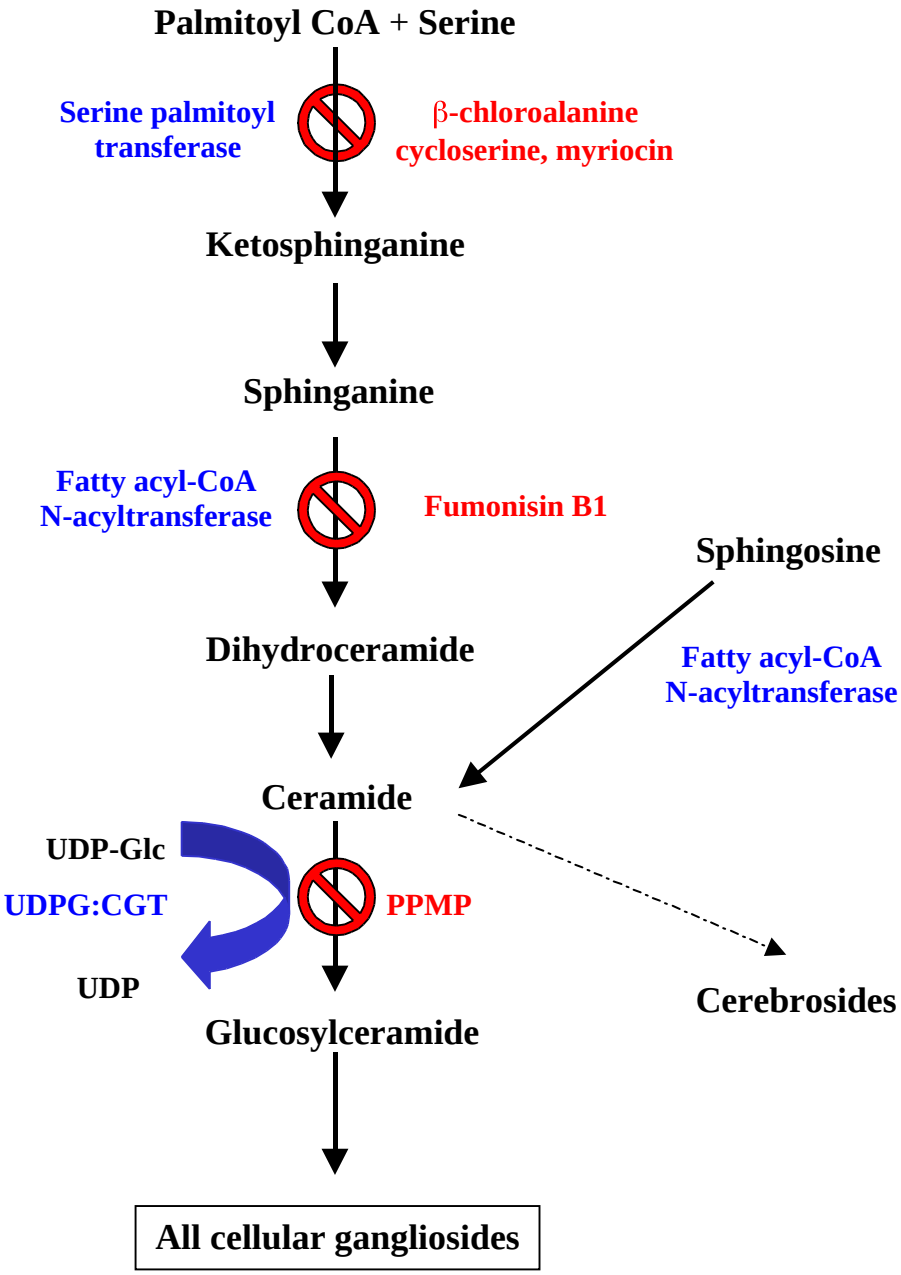

Fig. 2 Pathway of glycosphingolipid synthesis

Glucose/Oxygen regulated proteins

The glucose/oxygen regulated proteins (GRPs/ORPs) are a group of ubiquitous proteins constitutively expressed, but upregulated in cells cultured under glucose starvation and/or hypoxia as well as in ischemic tissues (11). The members of this protein family include the mitochondrial chaperone GRP75 and the ER chaperones GRP58/ERp57, ERp72/CaBP2, GRP78/Bip, GRP94/ERp99 and GRP170/ORP150 (11). These proteins facilitate the folding of newly synthesized proteins and are conserved from yeast to higher plants and mammals, suggesting essential functions. GRP75 is required for the import and folding of mitochondrial proteins but little is known about the control of its expression (12). GRP58 and ERp72 are members of the thioredoxin superfamily which participate in disulphide isomerization during the folding of proteins of the secretory pathway (13). GRP78 and GRP170 are part of the ER machinery that imports proteins into the ER, and together with GRP94 assist in the folding and assembly of secretory and membrane proteins (11). At least two of these proteins GRP78 and GRP170 are required for cell survival under hypoxia (14). In addition, calreticulin and GRP78 account for most of the Ca^{2+} buffering capacity of the ER and their overproduction protects different cell types from situations which induce Ca^{2+} overload (11, 15). Since hypoxia/reperfusion cause a Ca^{2+} dependent damage (16), the upregulation of these stress proteins is likely to be of importance for cell survival during ischemic insult (11). The overexpression of the ER resident GRPs and other chaperones under glucose starvation and/or hypoxia, is thought to be regulated by a pathway designated the Unfolded Protein Response (UPR) (17).

The Unfolded Protein Response

The ER contains a high concentration of soluble molecular chaperones and folding enzymes providing an optimal environment for proper folding of newly synthesized proteins of the secretory pathway. In response to the accumulation of unfolded proteins within the ER, eukaryotic cells from yeast to higher eukaryotes activate an intracellular signaling pathway from the ER to the nucleus known as the UPR (17). This results in an enhanced transcription of the ER resident chaperones. Thus,

eukaryotic cells are able to increase the folding capacity of the ER according to their needs (17). The target genes of the UPR possess in their promoters a specific activating sequence that directs their transcription upon induction of the pathway (17). Much of the progress in understanding this pathway comes from studies in *Saccharomyces cerevisiae*. In this organism, three key components of the pathway have been identified: the transmembrane protein Ire1p, which senses unfolded protein accumulation in the ER lumen and transmits the signal across the ER membrane; Hac1p, a basic leucine zipper, bZIP, which binds to the unfolded protein response element, UPRE, in the promoter of their target genes inducing their transcription; and RIg1p, tRNA ligase, which bridges activation of Ire1p and production of Hac1p in response to the accumulation of unfolded proteins within the ER (17).

Ire1p, is a transmembrane serine/threonine kinase whose N-terminal domain resides in the ER lumen (18). Upon accumulation of unfolded protein within the ER, Ire1p oligomerizes and trans-autophosphorylates via its cytosolic kinase domain (18). Active Ire1p has a specific extra-luminal endonuclease activity that initiates the splicing of HAC1 mRNA (18). In this way the splicing of HAC1 mRNA allows cells to synthesize the transcription factor Hac1p when they need to cope with the accumulation of unfolded proteins in the ER (17).

Although many aspects of the UPR pathway have been conserved during evolution, in mammals the UPR pathway (Fig. 3) is more diverse and complex than in yeast (19). The mammalian UPR has at least two distinct components: the first consists of the upregulation of expression of genes whose products promote protein folding in the ER and degradation of misfolding proteins. The second component is a rapid and transient attenuation of new protein synthesis that can be considered as an attempt to limit the load of the folding apparatus in the ER (19). Three ER transmembrane kinases that play roles in responding to ER stress have been identified: IRE1α and IRE1β, which are homologous to IRE1p over the entire length, and PERK/PEK (19). The latter kinase shares homology with the N-terminal lumenal domain of Ire1p, but lacks an endonuclease domain and instead contains a divergent kinase domain. The homology in the lumenal domains of PERK, IRE1α and IRE1β indicates that these three proteins use similar sensing mechanisms (19). Indeed, it has

been shown that they interact with GRP78 directly and are activated when they dissociate from it during accumulation of unfolded proteins within the ER (20). Overexpression of wild type IRE1α or IRE1β is sufficient to induce transcription of the ER resident GRPs (18). Mutant forms lacking the cytosolic effector domain show dominant-negative effects on the UPR, supporting their importance in the pathway (18). However, there is no evidence indicating that mammalian cells use a mRNA splicing system similar to yeast to produce a transcription factor to activate the UPR. Instead, another ER transmembrane protein, the bZIP transcription factor ATF6, appears to be important in the mammalian UPR (16). ATF6 is constitutively synthesized as a 90 KDa protein and under ER stress the 50 KDa cytosolic domain is proteolytically cleaved from the ER membrane (21). Then, it translocates to the nucleus, where it binds to the ER stress element, ERSE, in the promoters of UPR-responsive genes, inducing their transcription (19). In addition, Ire1p overexpression promotes ATF6 activation, and a dominant negative Ire1α mutant blocks ATF6 signaling, which suggests that the mammalian Ire1p functions upstream of ATF6 (22). The ER transmembrane protein kinase PERK/PEK plays a role in the control of translation. When PERK is activated it phosphorylates the general translation factor elF2-α inhibiting translation initiation (19). This results in down-regulation of overall protein synthesis thus preventing additional synthesis of proteins under conditions that do not allow proper folding (19). A hallmark of the mammalian UPR activation is the induction of the 29 kDa transcription factor CHOP/GADD153, which is not expressed at normal conditions and has been implicated in growth arrest and cell death (23). CHOP expression is induced independently by the activation of Ire1p, PERK, or ATF6 (24, 25). Thus, the UPR in mammals has both cytoprotective and pro-apoptotic functions, and can result in either cell survival or cell death, depending on the nature of the insult as well its severity and duration (Fig. 3)

***Bacterial phospholipases* C**

General aspects

In 1941 MacFarlane and Knight reported for the first time that a bacterial toxin, the *C. perfringens* α-toxin, has enzymatic activity as a phospholipase C (26). More

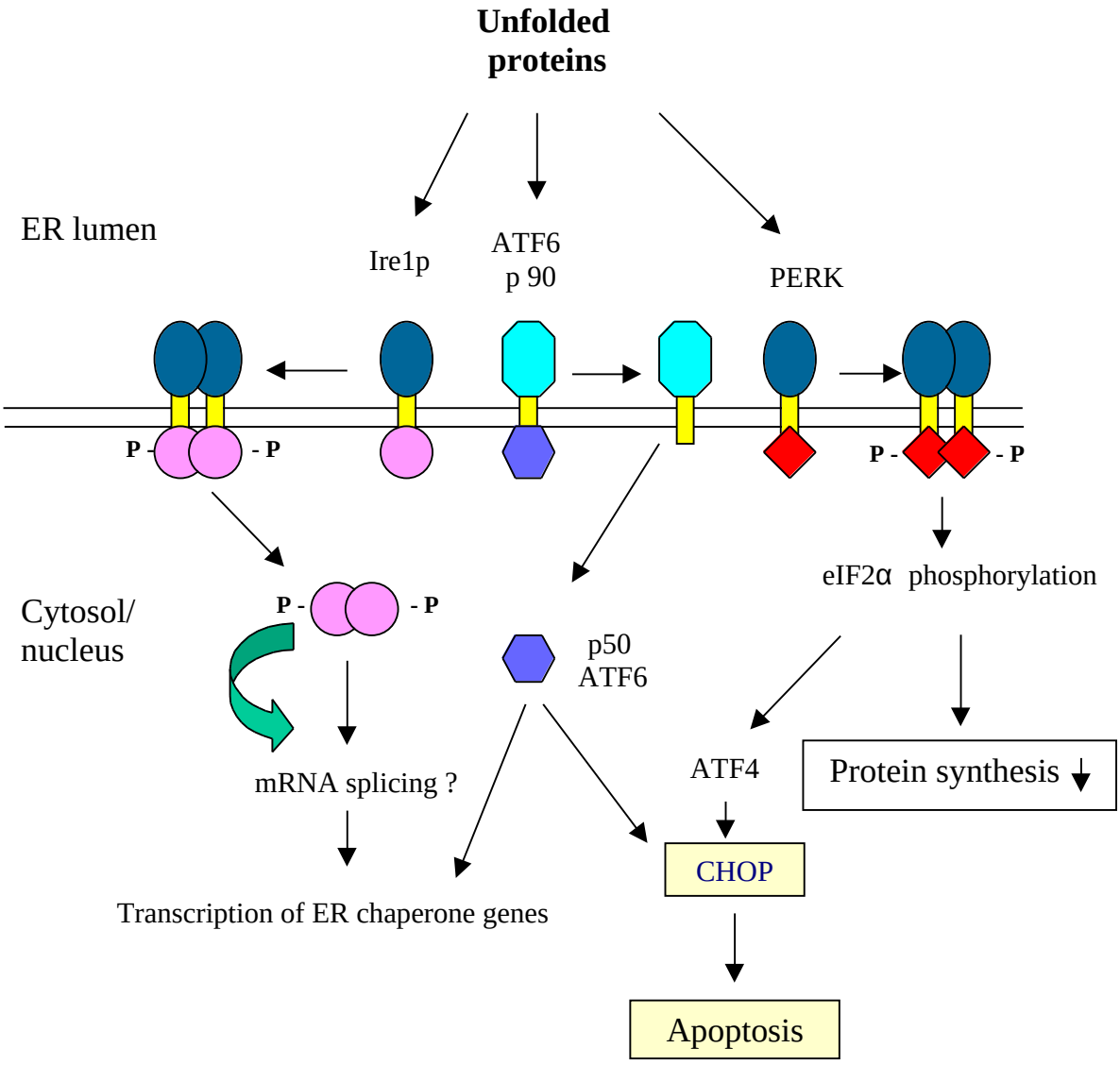

Fig. 3 The Unfolded Protein Response in mammalian cells

recently, many bacterial phospholipases have been characterized and found to be important virulence factors in human diseases (27). These enzymes are a diverse group of proteins able to hydrolyze phospholipids. They are classified as PLAs, PLCs or PLDs according to the site of phospholipid cleavage (28).

Bacterial phospholipases C have been classified into three groups according to whether the preferred substrate is phosphatidylcholine (PdtCho), SM or phosphatidylinositol (28). The PdtCho-preferring bacterial PLCs include Zn^{2+} metalloenzymes from *Bacillus cereus, Listeria monocytogenes* and several *Clostridium* sp. (28). They display different abilities to hydrolyze SM, have molecular weights in the range of 29-43 kDa, and show 30-60% amino acid sequence identity. Zn^{2+} metallophospholipases C contain three Zn^{2+} ions, which form part of their active sites (28). Based on their amino acid sequence phospholipases C can be classified into two groups: single domain proteins and two-domain proteins (28). The single domain proteins consist of approximately 250 amino acids. Members of this group are *Listeria monocytogenes* PLC and *Bacillus cereus* PC-PLC (28). The two-domain proteins are usually composed of 350 amino acids. These proteins are typified by the *Clostridium bifermentas* PLC and *C. perfringens* PLC (28). The N-terminal domain of the two domains phospholipases has an overall structural similarity with the single domain enzymes, as has been shown by crystallographic studies of *C. perfringens* PLC and *B. cereus* PC-PLC (29). One of the main biological differences between *C. perfringens* PLC and *B.cereus* PC-PLC is that the first one is toxic and able to lyse erythrocytes whereas the second is not, suggesting that the C-terminal domain plays a key role in this process (28). Other Zn^{2+} metallophospholipases C that have a C-domain are the *Clostridium novyi* γ−toxin and the *C. bifermentans* PLC which are also hemolytic (28).

Cellular effects of phospholipases C

Phospholipases C play important and in some cases key roles in the pathogenesis of disease (27). These enzymes function not only by causing wide scale cell lysis but also by causing limited hydrolysis of membrane phospholipids, the products of which can deregulate the cell metabolism (28). Central in this process is the generation of

diacylglycerol (DAG) from glycerophospholipids and ceramide from sphingolipids (28).

DAG is a substrate for diacylglycerol lipase, which produces arachidonic acid, that in turn, serves as a precursor in the production of prostaglandins, thromboxans and leukotrienes. These molecules have a potent proinflammatory activity and play a key role in the induction of platelet aggregation (28). DAG can also activate protein kinases C (PKC) within cells, and the active PKCs in turn activate eukaryotic membrane bound phospholipases (28). These activated phospholipases lead to further hydrolysis of membrane phospholipids or as in the case of PI-PLCs, generate inositol trisphosphate (IP3). IP3 acts as a second messenger which causes the release of calcium from the ER (28).

Ceramide is an important second messenger involved in the regulation of several signaling pathways including apoptosis, cell senescence, the cell cycle, and differentiation (30). Ceramide activates a number of kinases including stress activated protein kinases, such as the c-jun kinase as well as a kinase suppressor of Ras (30). Furthermore, ceramide can inhibit kinases such as PKCs and this inhibition appears to involve the ability of ceramide to activate protein phosphatases (30). While ceramide stimulates signal transduction pathways that are associated with cell death or cell arrest, DAG activates PKCs, which are associated with cell growth and cell survival. Thus ceramide and DAG generation may serve to monitor the cellular homeostasis by inducing pro-death or pro-growth pathways, respectively.

Clostridium perfringens

C. perfringens is a Gram-positive anaerobe which is ubiquitous in the environment, being found in the soil, in organic matter and as a member of the normal gut flora of many animals (31). *C. perfringens* is responsible for a variety of human and animal diseases including gas gangrene, food poisoning, necrotic enteritis and enterotoxemia (31). The bacterium is capable of producing a range of exotoxins and the differential production of these toxins is used to type different strains of the bacterium into one of five biotypes (A-E). Whilst biotype B-E strains are associated with disease in animals, biotype A strains are associated to the majority of human

infections (31). Of the many extracellular toxins produced by this bacterium the PLC and the theta toxin are the major virulence factors, and all strains possess the gene encoding the PLC.

C. perfringens is the widest spread of the histotoxic clostridia. The number of *C. perfringens* organisms carried by healthy humans has varied from 100 per g of stool among Swedish subjects to as high as $1x10^9$ per g of stool among some Japanese subjects (32). Under optimal conditions, its generation time can be as short as 8-10 minutes and its growth is accompanied by abundant gas production (32).

Biological activities and *structure of C. perfringens* PLC

The *C. perfringens* PLC, the most toxic among clostridial PLCs, displays platelet aggregating, hemolytic, myotoxic, and lethal activities (33). It has been shown that this toxin activates the arachidonic acid cascade and PKC (33). However, the mechanism by which PLC causes cell death has not been clarified.

This toxin is a zinc-metalloenzyme composed of 370 amino acids (Fig. 4). It was cloned by several groups in 1989 and its crystal structure was solved in 1998 (33). PLC shows two domains joined by a short hinge region (29). The N-terminal domain contains the active site and consists of 10 tightly packed α-helices where the three Zn^{2+} ions are located (Fig. 4). These Zn^{2+} ions are coordinated by tryptophan, glutamic acids, two aspartic acids and five histidine residues (29). Site directed mutagenesis of these residues results in variants which are devoid of enzymatic activity (34). A truncated version of the *C. perfringens* PLC containing only the N-terminal domain retains the lecithinase activity but lacks the sphingomyelinase activity, and it is not hemolytic (32).

The C-terminal domain is a β-sandwich composed of two four-stranded sheets, analogous to C2 domains of intracellular eukaryotic proteins involved in vesicular transport and signal transduction (29). The C-terminal domain is required for the Ca^{2+}-dependent interaction with aggregated PdtCho, and for the disruption of

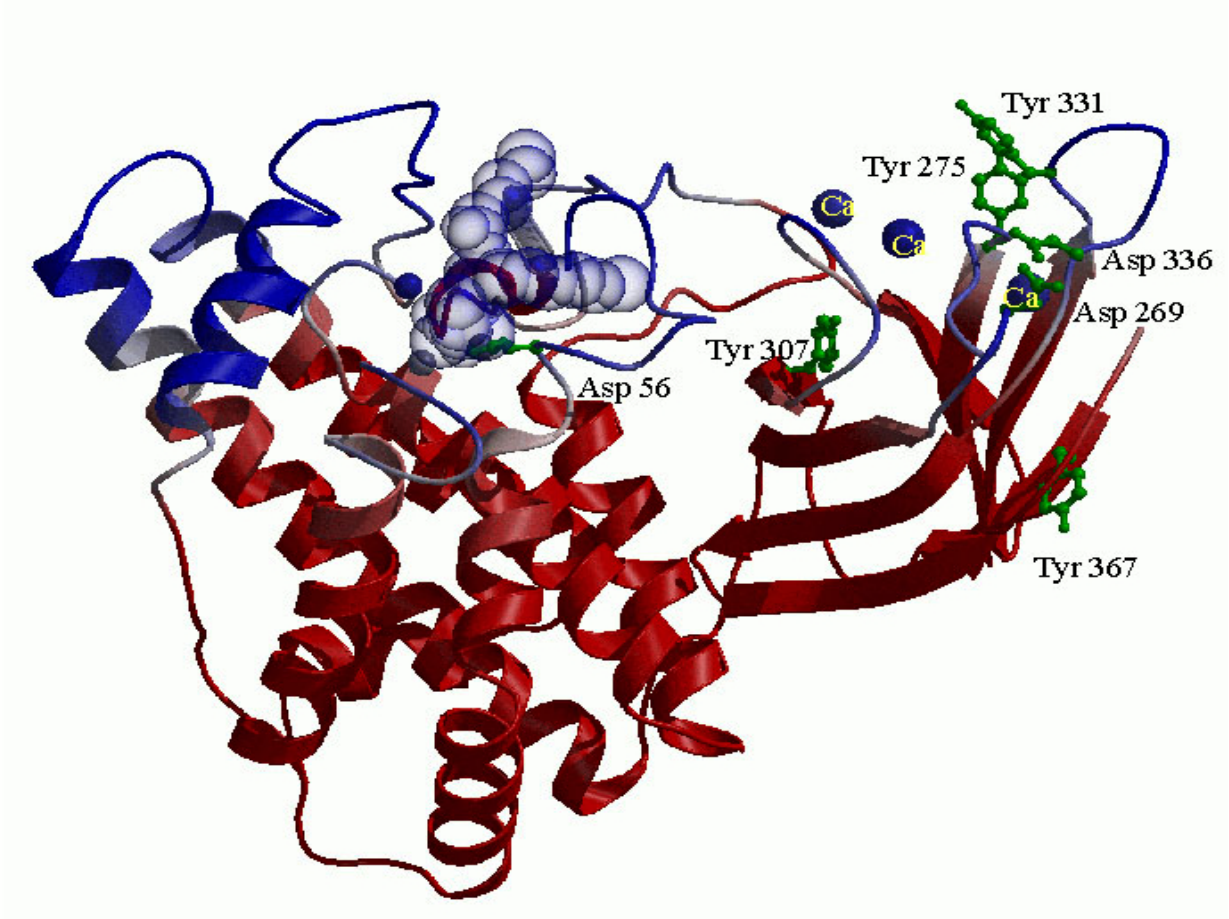

Fig. 4 Structure of *Clostridium perfringens*

artificial membranes (35). The amino acid residues involved in Ca^{2+}binding are 265-275, 292-303 and 330-339 and are thought to constitute part of the membrane-interacting surface, which facilitates the interaction of the toxin with membrane phospholipids (29). Mutation of Tyr275 and Asn269 residues result in proteins with reduced lechitinace activity (35). The N-terminal and C-terminal domains are joined by a flexible linker region, by interactions between the N-domain loop making contact with the C-domain, and by hydrophobic interaction between the adjacent faces of the domains (29). Crystallographic studies have revealed that *C. perfringens* PLC might exist in two conformations: an open form with the active site accessible and a closed form, with the active site covered by the loop encompassing residues 135 to 150 (36). It was suggested that the binding of the C-terminal domain to the target membrane causes the uncovering of the active site thus allowing hydrolysis of the phospholipid substrate (36).

Regulation of the C. perfringens plc gene expression

The gene encoding the PLC is chromosomically located within one of the most stable regions of the bacterial chromosome. The *C. perfringens* toxins production is regulated by a two-component signal transduction system that consists of a sensor histidine kinase, VirS and a response element VirR (31). The genes coding for these proteins are located in an operon on the *C. perfringens* chromosome, at a site quite distinct from the regions carrying the various toxin structural genes (31). Regulation of this system occurs at the transcriptional level. The mechanisms by which the activated VirR protein controls *C. perfringens* PLC production and the factors that stimulate the VirS/VirR regulatory cascade are unknown (31). It has been postulated that a small, intracellular signaling molecule produced by *C. perfringens*, may function as a quorum sensor controlling the expression of the VirS/VirR regulon (31). Once the concentration of *C. perfringens* reaches a certain density in host tissues, the level of this signal molecule may become sufficient to bind VirS, activate the regulatory cascade, and increase toxin production (31).

The plc gene possesses three A-tracts forming a sequence directed DNA curvature, bent, upstream of the promotor that have a stimulatory effect (36). This stimulatory

effect on the plc promotor is proportional to the number of A tracts and is more prominent at lower temperatures than at higher temperature (36). The bent DNA enhances formation of the RNA polymerase-plc promotor complex. (36). Thus, when living symbiotically *C. perfringens* produces low levels of PLC, as too much of it would be detrimental to the host. However, when present in the soil, or following the death of its host, the bacterium induces high level production of PLC which degrades cell membranes or other available lipids to generate energy to growth.

C. perfringens PLC and gas gangrene

Gas gangrene has been defined as an invasive, necrotizing infection caused by anaerobic bacteria and characterized by extensive local edema, massive tissue necrosis, variable degrees of gas production and profound toxemia (32). This infection is most frequently caused by *C. perfringens*, although other *Clostridium* species associated with the development of gas gangrene include *C. novyi*, *C. septicum*, *C. histolyticum*, *C. bifermentans*, *C. fallax*, and *C. sporogenes* (38,39). Trauma introduces bacteria into the deep tissues, and produces an anaerobic niche with a sufficiently low redox potential and acid pH for optimal clostridial growth. Gas gangrene is also an occasional consequence of surgery involving the intestinal tract (40). The infection can become well established in wounded tissues in 6-8 hours. The rapid progression of infection and tissue necrosis is related to the absence of an acute tissue inflammatory response, to tissue perfusion deficits resulting from toxin mediated vascular dysfunction and injury, and to the elaboration of potent cytotoxins and proteases (41). The destruction of adjacent healthy muscle can progress several centimeters per hour, and even with modern medical advances and intensive care regimens, the practice of radical amputation remains the single best treatment (42).

Two toxins of *C. perfringens*, PLC and theta toxin have been thought to contribute to the pathogenesis of gas gangrene via different mechanisms. However, several lines of evidence indicate that PLC in animal models is the major virulence factor in this disease (32). The active or passive immunization of animals against PLC protected them from gas gangrene (32). Furthermore, in experimental infections in mice with genetic mutants of *C. perfringens* lacking the plc gene, mortality was dramatically reduced (43).

The PLC induces a rapid decline in both mean pressure and cardiac index and has a direct cardiodepressent activity (41). Theta toxin induces a rapid drop in peripheral vascular resistance. Both toxins induce production of the vasodilator prostacyclin, and the platelet-activating factor by endothelial cells (41). Rapid progression of tissue destruction is the consequence of toxin effects on cells of the homeostatic system. PLC stimulates formation of intravascular aggregates of activated platelets, leading to a rapid and irreversible decline in muscle perfusion (42). It also induces synthesis of TNF by mononuclear cells. Both PLC and theta toxin induce expression of endothelial factors and leukocyte adherence molecules. Fulminant tissue destruction characteristic of clostridial gas gangrene is related to profound attenuation of local and regional blood flow, mediated by the effects of PLC on endothelial cells and platelets, and sustained by fibrin formation and hyperadhesion of leukocytes to the vascular endothelium (41).

The conspicuous absence of leukocytes and anti-inflammatory response at the site of *C. perfringens* proliferation is a hallmark of gas gangrene. It has been hypothesized that both *C. perfringens* PLC and theta toxin dysregulate the normal physiologic mechanisms of leukocyte accumulation, adherence and extravasation (41). A high concentration both toxins are cytolytic for leukocytes (41). In cultured endothelial cells PLC strongly induces the expression of E-selectin and ICAM-1, that appears to be related with the accumulation of neutrophils in the blood vessels (41). This toxin also stimulates production of endothelial cell derived IL-8, that could amplify the recruitment of leukocytes and prime them for enhanced respiratory burst activity. It has been reported that neutrophils exposed to high concentrations of IL-8 lose the ability to transmigrate through an endothelial cell monolayer in response to different chemoattractants (41). Finally, increased expression of PMNL adhesion molecules such as CD11b/CD18 could result in reduced diapedesis. These dysregulated events are associated with a profound attenuation of local and regional blood flow that could lead to ischemia, thereby favoring optimal clostridial proliferation. As infection progresses, large venous channels will become affected, causing regional vascular compromise, and necrosis. When toxin reaches the arterial circulation, systemic shock, multiorgan failure and death occur (41).

AIMS

The aims of this work were to clarify:

i) The reason for the low level of UDP-Glc in a Chinese Hamster mutant cell line

ii) Some consequences of a cellular UDP-Glc deficiency

iii) The role of UDP-Glc deficiency in the cellular sensitivity to *C. perfringens* PLC

RESULTS AND DISCUSSION

A mutant cell line deficient in UDP-Glc

Isolation of a revertant cell line

The Chinese hamster lung fibroblast mutant Don Q, which has a permanent UDP-Glc deficiency (44) was previously found to be 10^5 times more sensitive to *C. perfringens* PLC (45). To generate a cell line resistant to this PLC, Don Q was cultured in the presence of PLC. A spontaneous revertant cell line (Don QR) which regained the relative resistance to the *C. perfringens* PLC was isolated (Fig.1 paper III). The level of UDP-Glc in Don QR was found to lie between the level found in Don wt and Don Q (Table I, paper I), indicating that this cell line partially compensated the UDP-Glc deficiency.

The UDP-Glc deficiency is due to a point mutation

The synthesis of UDP-Glc depends on the glucose uptake by specific glucose transporters in the plasma membrane, and on the amount of UTP available in the cell. To clarify the reason for the low level of UDP-Glc in Don Q the uptake of glucose and its metabolism through the pathway of UDP-Glc synthesis (Fig. 1) was studied in the Don Q, wt and QR cells. The glucose uptake measured as incorporation of the glucose analogue 2-deoxyglucose was not decreased in Don Q, compared to Don wt and QR (Fig. 3, paper I). The levels of Glc-6-P and UTP were found to be very similar among the three cell lines (Table I, paper I). However, the measurement of the metabolites involved in the pathway of synthesis of UDP-hexoses showed that the mutant cell line has higher levels of Gal-1-P and Glc-1-P, and lower levels of UDP-GlcUA, UDP-Gal, and UDP-Glc than Don wt and QR (Table I, paper I). UDP-Glc is needed in the synthesis of UDP-Gal, which explains the lower level of this metabolite and the higher amount of its precursor Gal-1-P, in Don Q compared to Don wt and QR (Fig. 1). The increased level of Glc-1-P in Don Q and the low level of UDP-Glc suggested an impaired synthesis of UDP-Glc in this step. For that reason the activity of UDPG:PP, the enzyme that interconverts these metabolites was measured. Don Q

showed 4%, and Don QR showed 56% of the UDPG:PP activity found in Don wt (Table II, paper I). These results indicated that Don Q has an impaired capacity to synthesize UDP-Glc, which has been partially compensated in Don QR. The lowered UDPG:PP activity in Don Q and QR could result from a decreased gene transcription, an increased mRNA degradation, or a mutation in the coding region of the gene. The amount of UDPG:PP mRNA was similar in the three cell lines, as determined by semiquantitative RT-PCR and Northern blot analysis. Furthermore, the amount of the UDPG:PP protein was also similar in the three cell lines as determined by Western blot analysis. These results suggested that the impaired activity of UDPG:PP is not due to a decreased gene transcription or translation or to an increased mRNA or protein degradation. In order to determine whether there was a mutation in the coding region of the gene associated with the low enzymatic activity, the complete nucleotide sequence of the UDPG:PP cDNA from the three cell lines was established. Sequence analysis in Don Q showed a single point mutation, a guanine to adenine transition at position 347 of the coding sequence. In Don QR an equimolar mixture of guanine and adenine at position 347 was found. This suggested that the mutation is present in homozygous form in Don Q and is reverted in only one of the alleles in Don QR. This mutation changes a glycine residue located at position 115 to aspartic acid (Fig. 5, paper I). This glycine residue is strictly conserved among eukaryotic UDPG:PPs and predictions of the secondary structure of the UDPG:PP indicated that it is located in a reverse turn (Fig. 5, paper I). It was concluded that the G115D substitution dramatically impairs the enzymatic activity of the protein product, leading to a persistently low UDP-glucose level in Don Q.

The UDP-Glc deficiency causes the overexpression of stress proteins

In *E. coli* a low UDP-Glc level seems to be a signal that induces the expression of a set of stress proteins required for survival under adverse conditions. Similarly in plant cells a low UDP-Glc level induces the synthesis of at least one stress protein during exposure to hypoxia or glucose starvation. Therefore, Don wt, Q and QR were used to determine whether the UDP-Glc deficiency induces the overproduction of stress proteins in mammalian cells.

To determine whether the mutant Don Q has any specific changes in the cellular protein pattern, whole-cell lysates of Don wt, Q and QR cells were analyzed by two-dimensional gel electrophoresis (Fig. 1, paper II). Several protein spots were consistently detected in higher amounts in Don Q than in Don wt and QR cell lysates. These overproduced proteins were identified by their N-terminal amino acid sequences as GRP58/ERp61/ERp57, calreticulin (CRT), GRP75/PBP74/mortalin, and GRP78/BiP/ORP80, respectively (Table 2, paper II). Western blot experiments further confirmed the identity of the overproduced proteins and showed that they occur in about 3 times higher relative amounts in Don Q than in Don wt and QR cells (Fig. 3, paper II). Accordingly, reporter assays showed that the promoters of the GRP78, GRP94, GRP58, Erp72 and CRT are from 2.3-to 3.8 times more active in QC than in B9 cells (Fig. 4 paper II). These results indicate that the overexpression of these chaperones is due to an increase transcription. For that reason, additional Western blot analyses were performed with antibodies against other members of this protein family: ERp72/CaBP2, GRP94/ERp99/endoplasmin, and GRP170/ORP150. The relative amounts of these proteins were also about 3 times higher in Don Q than in the Don wt and QR cells (Fig. 3, paper II). These proteins are residents of the ER (GRP58, GRP78, GRP94, and caleticulin) or the mitochondria (GRP75) and belong to the stress-inducible GRP/ORP family. They are constitutively produced by the cell at a basal level, but the overproduction of ER resident GRPs has been related to the accumulation of misfolded proteins in the ER. However, Western blot analysis revealed that Don wt, Q and QR produce similar relative amounts of other ER stress-inducible proteins (UDP-Glc: glycoprotein glucosyltransferase, the peptidyl-prolyl *cis-trans* isomerase CPH2, the protein disulphide isomerase as well as the antioxidant enzyme heme oxygenase 1/HSP32). Moreover, the cytosolic HSP70 and HSP25, the mitochondrial HSP60, as well as the Cu^{2+}/Zn^{2+} superoxide dismutase were found in comparable amounts in the three cell lines. This showed that only a specific set of stress proteins are induced in Don Q, and suggested that this induction apparently is not caused by accumulation of misfolded proteins in the ER.

Don Q cells were transfected with the bovine UDPG:PP cDNA in order to restore the cellular level of UDP-Glc in this cell line. Several clones were isolated of which B9 and G3 are representatives. ^{1}H-NMR measurements showed that the UDP-Glc concentration in these transfectant clones was comparable to that found in Don wt or

QR cells (Table I, paper II). The relative amounts of calreticulin and the six GRPs/ORPs in the B9 and G3 transfectants were from 2.3 to 3.4 times lower than in the UDP-Glc deficient control QC as compared by Western blot analysis.

It was found that under normal growth conditions Don wt, QR or G3 cells did express p90ATF6 but not p50ATF6, did not express CHOP, and produced GRP78 at a basal level (Fig. 5, paper II). Exposure of these cells to tunicamycin triggered the UPR, the conversion of p90ATF6 to p50ATF6, induced the expression of CHOP, and increased about 3 fold the amount of GRP78 (Fig. 5, paper II). These results indicated that the upregulation of ER resident chaperones in these cells is independent of ATF6 activation and demonstrated that the UPR remains functionally intact. The findings that in Don Q/QC cells, several ER resident folding enzymes are not overproduced, CHOP is not expressed, p50ATF6 is not present and the mitochondrial GRP75 is coordinately overproduced suggested that the signaling pathway triggered by the UDP-Glc deficiency is molecularly distinct from the UPR. Furthermore the finding that in Don Q/QC cells both CHOP and p50ATF6 were inducible by the exposure to tunicamycin showed that the UPR pathway remains functionally intact. This suggested that a UDP-Glc deficiency might constitute a stress signal which induces the overproduction of calreticulin and several chaperones of the GRP/ORP family in mammalian cells.

The UDP-Glc deficiency causes hypersensitivity to C. perfringens PLC

Previous data show that Don Q and wt cells exhibit the same sensitivity to phospholipases A2, B, and D, whereas Don Q is hypersensitive to the cytotoxic effect of *C. perfringens* PLC (45). To determine whether Don Q is hypersensitive to others PC-hydrolyzing phospholipases C, the sensitivity of Don wt, Q, and QR to two other phospholipases C was studied. The three cell lines showed the same sensitivity to *B. cereus* PLC, and *C. bifermentans* phospholipase C (Fig. 1, paper III). These data suggested that Don Q is specifically hypersensitive to the *C. perfringens* PLC. To clarify whether there is a connection between the UDP-Glc deficiency and the hypersensitivity to *C. perfringens* PLC, stable transfectant cells were prepared using a wild type UDPG:PP cDNA. Clones of the mutant cell transfected with a construct having the insert in the sense orientation had increased their UDP-Glc level. Clones of the revertant transfected with a UDPG:PP antisense had reduced their level of

UDP-Glc compared with control clones (paper III). Exposure of these two types of transfectant clones to *C. perfringens* PLC demonstrated that a cellular UDP-Glc deficiency causes hypersensitivity to the cytotoxic effect of this phospholipase.

Structure/function studies of C. perfringens PLC

The C-terminal domain and the sphingomyelinase activity are required for cytotoxicity

The hypersensitivity of Don Q to *C. perfringens* PLC made it an ideal tool to study the cytotoxic effect induced by this phospholipase and to map which parts of the toxin are relevant for its cytotoxicity.

The cytotoxic effect of a truncated version of the *C. perfringens* PLC (containing the 249 N-terminal residues) which retains the lecithinase activity but lacks the sphingomyelinase activity was determined in Don Q. This truncated toxin had a cytotoxic potency 10^6 times lower than the wild type enzyme (Fig. *7*A). This demonstrated that the C-terminal domain and the sphingomyelinase activity are required for the cytotoxic effect of this PLC and also showed that the lecithinase activity is not sufficient to confer cytotoxicity.

Polyclonal antibodies against the C-domain of *C. perfringens* PLC prevented the cytotoxic effect of the holotoxin in Don Q cells in a dose-dependent manner (Fig. *7*B, paper IV), demonstrating that this domain is crucial for cytotoxicity. In addition, the cytotoxic effect was potentiated about 10^2 times when the truncated PLC and the C-terminal domain where added simultaneously (Fig. *7*A, paper IV). However, a hybrid protein containing the C-terminal domain of *C. perfringens* PLC with the N-terminal domain of *C. bifermentans* PLC showed a cytotoxic potency 10^3 times lower than that of the *C. perfringens* PLC (Fig. 8C, paper IV). This result excluded the possibility that a simple complementation of the lecithinase with the C-terminal domain would suffice to confer full cytotoxicity. Furthermore it also suggested that a specific interaction between the two toxin domains is needed for full cytotoxicity.

Studies in Don Q were performed with enzymatically inactive *C. perfringens* PLC variants in which replacement of His-11 or His-68 by Ser, or Asp-56 by Asn resulted in a loss of the enzymatic activities. These His residues are involved in the coordination of Zn^{2+} ions, whereas Asp-56 is exposed on the surface within the active site cleft (29). Replacement of Asp-56 abolishes the lecithinase and sphingomyelinase activities without affecting the toxin membrane-binding capacity (33). All these three PLC variants were 10^4-10^5 times less cytotoxic to Don Q cells than the recombinant wild type PLC (Fig. 6B, paper III). These results therefore demonstrated that the catalytic capacity of the *C. perfringens* PLC is required for its cytotoxic effect.

The C-terminal domain and the sphingomyelinase activity are required for myotoxicity

Although it is known that *C. perfringens* PLC induces extensive myonecrosis there were no previous structure/function studies regarding the myotoxic activity. We showed that the wild type toxin induces a 35-fold increase in plasma levels of CK activity compared to its control. In contrast *C. bifermentans* PLC only produced less than 2-fold increase in the plasma level of CK. The variant Asp56Asn failed to induce a significant myotoxic effect (Fig. 4B, paper IV), indicating that the catalytic activity of *C. perfringens* PLC is required for its myotoxicity. The truncated version of the toxin encompassing its N-terminal residues induced a 50-fold lower increase in plasma CK activity than the wild type toxin (Fig. 4B, paper IV), indicating that the lecitinase activity *per se* is not sufficient to confer myotoxicity and that the C-terminal domain is required for full myotoxic activity.

Aspartic acid and tyrosine residues of PLC are important for toxicity

Several residues were selected for mutagenesis: Asp 269, Asp336, Tyr275, and Ty311, located in the putative membrane binding region of PLC, and Tyr307, which is located in interface between the N-terminal and C-terminal domains. The introduced residues were chosen on the basis of their propensity to adopt the same secondary structure as those of the corresponding wild type residues. Asp residues were substituted by Asn, to evaluate the role of carboxylate groups, whereas Tyr residues were replaced by Phe to determine the role of hydroxyl groups. The residues

Tyr275 and Tyr331 were substituted by Asn and Leu respectively, to mimic locally the structure of the *C. bifermentans* PLC.

The variants Asp269Asn and Asp336Asn showed less than 20% of the hemolytic activity (Fig. 2A, paper IV) and displayed a cytotoxic potency 10^3 fold lower than that of the wild type toxin (Fig. 2B, paper IV). The variants in which Tyr275, Tyr307, and Tyr331 were substituted by Asn, Phe or Leu had 11-73% of the hemolytic activity (Fig. 3A, paper IV) and exhibited a cytotoxic potency 10^2-10^5 fold lower than that of the wild type toxin (Fig. 3B-D, paper IV). The results demonstrated that the phenolic ring of Tyr275 is crucial for the hemolytic and cytotoxic activities and that the hydroxyl group at 275 is also important for those activities. The results obtained with the variants Tyr331Phe and Tyr331Leu showed the importance of the hydroxyl group at 331 for toxicity. The lowered hemolytic and cytotoxic activities of the variants Asp269Asn, Asp336Asn and Tyr307Phe are likely explained by a reduced capacity to interact with the membrane of the target cells and, in the case of Tyr307Phe, by a reduced capacity to communicate such interaction to the active site. In addition, the variants Asp269Asn, Asp336Asn, Tyr275Asn and Tyr331Leu which showed the lowest hemolytic and cytotoxic activities were selected for studies of myotoxicity. These variants showed less than 12% of the myotoxic potency of the wild type toxin (Fig. 5, paper IV).

The amphipathic side chains of tyrosine and tryptophan, make these residues ideally suited to reside in the complex electrostatic environment of the bilayer interface. Tyrosine and tryptophan residues have been suggested to play a critical role in the interaction of peptides with the interfacial region of lipid bilayers. The results of this work indicate that Tyr275 and Tyr331 are important for the interaction of the *C. perfringens* PLC with the cellular membrane of target cells. Since these tyrosine residues are Asn and Ile, respectively, in the *C. bifermentans* PLC, these substitutions could explain, at least partially, the lower hemolytic, cytotoxic and myotoxic activities of this enzyme in comparison with *C. perfringens* PLC

Role of glycoconjugates in the cell sensitivity to C. perfringens PLC

Since UDP-Glc is required in the synthesis and processing of proteoglycans, N-linked glycoproteins, and glycosphingolipids, the importance of these molecules in the cellular sensitivity to *C. perfringens* PLC was studied.

Don wt and the Chinese Hamster ovary cells (CHO) showed the same sensitivities to *C. perfringens* PLC, after pretreatment with chlorate, an inhibitor of processing and secretion of proteoglycans (Fig. 2 A,B paper V). Furthermore, a proteoglycan deficient CHO mutant cell line (pgs-110) exhibited the same sensitivity to PLC as control cells (Fig. 3, paper V). It was concluded that a deficiency in proteoglycans does not affect the cellular sensitivity to *C. perfringens* PLC.

Don wt and CHO cells pretreated with 1-deoxynojirimycin, an inhibitor of glucosidase I, or castanospermine, an inhibitor of glucosidases I and II, showed the same sensitivity to PLC as their controls (Fig. 4C-D, paper V). It was concluded that a specific inhibition of glycoprotein processing does not affect the cellular sensitivity to *C. perfringens* PLC.

Don wt cells were pretreated with myriocin, L-cycloserine or β-chloroalanine, inhibitors of *de novo* pathway of ceramide synthesis. Pretreated cells were about 10^2 times more sensitive to the cytotoxic effect of PLC than control cells (Fig. 5A-C, paper V). Pretreatment with fumonisin B1, which blocked both ceramide synthesis *de novo* and the salvage pathway induced a higher sensitization (10^4 fold) (Fig. 5D, paper V). Furthermore, pretreatment of cells with DL-threo-1-phenyl palmitoylamino-3morpholino1propanol (DL-PPMP), an inhibitor of the UDP-glucose:ceramide glucosyltransferase (UDPG:CGT), also increased their sensitivity to PLC about 10^4 times (Fig. 5E, paper V). Similar results were obtained with the same pretreatments in three other cell lines (CHO, HEp-2, and B16), showing that the effect of ganglioside synthesis inhibition in the cell sensitivity to *C. perfringens* PLC is not cell specific. In contrast, none of these GSL synthesis inhibitors affected the sensitivity of those cell lines to the *B. cereus* phospholipase C, indicating that the lack of GSLs sensitizes cells specifically to *C. perfringens* PLC.

The mouse mutant melanoma cell line (GM-95) has a defective ganglioside synthesis due to a mutation in the gene encoding the UDPG:CGT, which catalyses glucoceramide synthesis (Fig. 1, paper V). Interestingly, the GM-95 cells were 10^5 times more sensitive to PLC than control cells (Fig. 6A, paper V), although they exhibited the same relative resistance to the *B. cereus* phospholipase C (Fig. 6B, paper V). These results demonstrate that a cellular ganglioside deficiency specifically causes hypersensitivity to the cytotoxic effect of *C. perfringens* PLC.

Complex gangliosides protect sensitive cells from the effect of PLC

Don Q and GM-95 cells preincubated with a ganglioside mixture were protected in a dose-dependent manner from the cytotoxic effect of PLC (Fig. 7A, paper V). In contrast, pretreatment of Don Q or GM-95 cells with a mixture of cerebrosides (monosaccharide-containing GSLs) did not have any protective effect (Fig. 7B, paper V). Pretreatment of Don Q with the simple gangliosides (GM3 or asialo GM1) did not protect from the cytotoxic effect of PLC (Fig. 9, paper V). However, pretreatment with complex gangliosides of the a series (GM1a or GD1a) or the b series (GD1b or GT1b), increased the cell resistance to PLC in a dose-dependent manner (Fig. 9, paper V). The protective effect increased with the size of the oligosaccharide chain of the polar head group, indicating that multiple SiaAc units of complex gangliosides play a protective role. Accordingly, the pretreatment of Don wt, or HEp-2 cells with neuraminidase increased their sensitivity to the cytotoxic effect of PLC in a dose-dependent manner (Fig. 10A-B, paper V).

The degradation of membrane phospholipids and the disruption of the cellular membrane in cells with different UDP-Glc levels was measured. The *C. perfringens* PLC induced a higher release of labeled choline from the membrane of prelabeled Don Q than from Don wt or QR cells (Fig. 11A-B, paper V). Furthermore, it also induced a higher release of lactate dehydrogenase (LDH) from Don Q and GM-95 than from the their transfectant counterparts. In contrast, *B. asper* myotoxin II, a membrane disrupting toxin with broad cytolytic specificity, induced a similar LDH release from Don Q and GM-95 as from their transfectant controls (Fig. 12B, paper V). These results indicated that the hypersensitivity of Don Q and GM-95 cells to *C. perfringens* PLC depends largely on a facilitated disruption of the cellular membrane.

Complex gangliosides protect liposomes from the effect of PLC

The influence of ganglioside incorporation on the susceptibility of artificial membranes made of PC or SM to disruption by *C. perfringens* PLC was studied. Addition of the complex gangliosides GM1a, GD1a, GD1b or GT1b prevented the membrane disruption caused by PLC in a dose-dependent manner (Fig. 13A-B, paper V). In contrast, addition of the simple gangliosides GM3 or asialoGM1 did not protect liposomes from the membrane disrupting effect of PLC (Fig. 13A-B, paper V).

In conclusion, this work provided new insights toward understanding the factors involved in the cell susceptibility to *C. perfringens* PLC. The results showed that a lack of gangliosides renders cells more sensitive to PLC. In contrast, their presence in the plasma membrane of the target cell or artificial membranes confers relative resistance to this toxin. It was also concluded that the increased susceptibility to PLC in UDP-Glc deficient cells is related to an increased degree of phospholipid hydrolysis, which leads to disruption of the plasma membrane.

CONCLUDING REMARKS

This work showed that UDP-Glc induces the upregulation of a set of stress proteins involved in cell survival and the regulation of calcium homeostasis. It has been reported that a low UDP-Glc concentration occurs in cells cultured under low glucose concentration, or hypoxia. The expression of these stress proteins is down regulated in Alzheimer disease and this is associated to an increased susceptibility to cell death induced by disturbances in calcium homeostasis. The overexpression of the stress proteins of the GRPs/ORPs family is induced in ischemic tissues where they likely play a protective role. Solid tumors contain a deficient blood supply due to their abnormal vasculature. Indeed an overexpression of the GRPs/ORPs is often triggered in tumor cells and this has been associated to resistance to radiotherapy and chemotherapy. Understanding the pathway that controls the upregulation of these stress proteins in cells under a UDP-Glc deficiency might facilitate the identification of new targets for novel treatments of these diseases

This work also demonstrated that the UDP-Glc deficiency impairs the synthesis of gangliosides, inducing changes on the plasma membrane which lead to an increased sensitivity to *C. perfringens* PLC, the major virulence factor in gas gangrene. Complex gangliosides may interact directly with the PLC preventing it from reaching its substrates. Alternatively, gangliosides might compete in the binding of the toxin with a putative receptor, preventing in this way its mode of action. Therefore these findings offer a possible explanation for the high susceptibility of muscle fibers to the cytotoxic effect of PLC, since muscle cells are known to have the lowest concentration of complex gangliosides among all mammalian tissues studied. The fact that *C. perfringens* only can grow in ischemic tissues and that the PLC could be more cytotoxic in these tissues opens the possibility of using the UDP-Glc deficient cell line as a model to elucidate the molecular mechanism of the cytotoxic action of *C. perfringens* PLC.

SUMMARY IN SPANISH

Una línea celular de Fibroblastos de Hamster Chino, deficiente de UDP-Glc e hipersensible a la fosfolipasa C de *Clostridium perfringens* (PLC) fue usada en este trabajo para determinar algunas de las consecuencias moleculares de la deficiencia de UDP-Glc. Además, para realizar estudios estructura/función que permitieron identificar residuos críticos para la actividad citotóxica de la PLC, el principal factor de virulencia en la patogénesis de gangrena gaseosa.

Se determinó que la razón de la deficiencia de UDP-Glc en esta célula es una mutación puntual en el gen de la UDP-Glc pirofosforilasa (UDPG:PP), la enzima que cataliza la formación de UDP-Glc. Además se encontró que la línea celular mutante sobreproduce siete proteínas de estrés: una chaperona mitocondrial (GRP75) y seis chaperonas del retículo endoplásmico (GRP58, ERp72, GRP78, GRP94, GRP170 y calreticulina). Estas proteínas son sobreproducidas en células cultivadas en hipoxia, baja concentración de glucosa y en tejidos isquémicos. Para clarificar si existe una conexión entre la deficiencia de UDP-Glc, la sobreproducción de proteínas de estrés y la hipersensibilidad a la PLC, la célula mutante fue transfectada usando cDNAs de la UDPG:PP silvestre. Los clones transfectados aumentaron su concentración de UDP-Glc y produjeron cantidades normales de calreticulina y de las GRPs, demostrando que la deficiencia de UDP-Glc induce la sobreproducción de estas proteínas. Exposición de los clones transfectados a la PLC demostró que la deficiencia celular de UDP-Glc causa hipersensibilidad a esta fosfolipasa.

La línea celular deficiente de UDP-Glc caracterizada en este estudio fue usada en estudios que permitieron identificar los determinantes estructurales responsables de la actividad citotóxica de esta PLC. Experimentos con variantes generadas por ingeniería genética demostraron que la actividad esfingomielinasa y el dominio C-terminal son requeridos para la actividad citotóxica . Además experimentos *in vivo* indicaron que la actividad esfingomielinasa y el dominio C-terminal son también necesarios para la actividad miotóxica de la PLC. Las actividades tóxicas de variantes con una sola sustitución en residuos de ac. aspártico los cuales unen calcio o en residuos de tirosina del dominio C-terminal, que se postula interaccionan con membranas, fueron estudiados. Estos residuos son críticos para las actividades hemolítica, citotóxica y miotóxica de la PLC.

Como la UDP-glucose es un precursor en la síntesis de glicoconjugados de membrana, su papel en la sensibilidad a la PLC fue estudiada. Se encontró que la inhibición de la síntesis y/o procesamiento de glicoproteínas y proteoglicanos no afecta la sensibilidad de las células a esta toxina, mientras que la inhibición de la síntesis de glicolípidos las sensibiliza. Además, se encontró que gangliosidos complejos protejen células hipersensibles a la PLC de su efecto citotóxico y previene la ruptura de membranas artificiales inducida por esta toxina.

En conclusión, este trabajo revela que la deficiencia celular de UDP-Glc induce la sobreproducción de un grupo de proteínas de estrés importante para la sobrevivencia celular en condiciones de isquemia y además provee nuevo conocimiento para entender el mecanismo de acción de la PLC a nivel molecular.

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