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Localisation of Protein Kinase C in Apoptosis and Neurite Outgrowth

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Academic dissertation
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Abstract

Protein kinase C (PKC) is a family of serine/threonine kinases, which are subgrouped into classical (α, βI, βII, γ), novel (δ, ε, η, θ) and atypical (ζ, ι/λ) isoforms. One major aim of this thesis work was to investigate if altered levels of PKC isoforms influence the apoptotic responses of malignant cell-lines. We show that overexpression of PKCδ or PKCθ renders SK-N-BE(2) neuroblastoma cells sensitive to apoptosis induced by phorbol esters or C2-ceramide. Moreover, overexpression of PKCα, PKCδ or PKCε sensitises both androgen-dependent and androgen-independent prostate cancer cells to phorbol ester-induced cell death. The apoptotic effects of PKCδ and PKCθ are independent on the catalytic activity of the enzymes and the isolated regulatory domain (RD) of PKCθ induces apoptosis in neuroblastoma cells. Induction of apoptosis depends on the localisation of PKCθRD to the Golgi complex, which is mediated by the C1b domain of the protein. Mutation of a single amino acid residue, Met-267 in PKCθC1b, blocks both the Golgi localisation and the apoptotic effect of PKCθRD.

Previous studies have shown that PKCε induces neurites in neuroblastoma cells. Here we report that treatment with cell-permeable C2-ceramide inhibits PKCε-induced neurite formation, conceivably by relocating the protein from the cytosol to the perinuclear region. Mutation of Asp-257 and Met-278 (the latter corresponding to Met-267 in PKCθ) in PKCε blocks the C2-ceramide induced translocation of PKCε. Furthermore, the mutated variant of PKCε still induces neurites after C2-ceramide treatment. Thus, the specific subcellular localisation of PKCθ and PKCε are important for their biological activities.

Key words Protein Kinase C, regulatory domain, C1 domain, apoptosis, neurite outgrowth, Golgi apparatus, neuroblastoma, prostate cancer, ceramide

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This thesis is based on the following papers, which are referred to as Papers I-IV:

I. **Anna Schultz**, Jan-Ingvar Jönsson and Christer Larsson. The regulatory domain of protein kinase C-θ localises to the Golgi complex and induces apoptosis in neuroblastoma and Jurkat cells. Cell Death. Differ. 2003;10:662-675.

II. **Anna Schultz** and Christer Larsson. Ceramide influences neurite outgrowth and neuroblastoma cell apoptosis regulated by novel protein kinase C isoforms. J. Neurochem. 2004; 89:1427-1435.

III. **Anna Schultz**, Mia Ling and Christer Larsson. Identification of an amino acid residue in the protein kinase C C1b domain crucial for its localization to the Golgi network. J. Biol. Chem. 2004; 279:31750-31760.

IV. **Anna Schultz** and Christer Larsson. Protein kinase C-ε induces cell death both in androgen-dependent and androgen-independent prostate cancer cells. Manuscript.

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ABBREVIATIONS

APAF-1	apoptotic protease activating factor-1
AR	androgen receptor
BH	Bcl-2 homology
Bcl-2	B-cell lymphoma-2
CD	catalytic domain
CAM	cell adhesion molecule
DAG	diacylglycerol
DD	death domain
DISC	death-inducing signalling complex
DNA-PK	DNA-dependent protein kinase
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain
FL	full-length
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitors
GEF	guanine nucleotide exchange factors
GTP	guanosine triphosphate
IAPs	inhibitors of apoptosis
JNK	c-Jun terminal kinase
KO	knock-out
LH-RH	luteinising hormone releasing hormone
MAPK	mitogen-activated protein kinase
MMP	mitochondrial membrane permeabilisation
NGF	nerve growth factor
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PDGF	platelet derived growth factor
PDK-1	phosphoinositide dependent kinase-1
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
PLD	phospholipase D
RA	retinoic acid
RACK	receptor for activated C kinase
Rb	retinoblastoma
RD	regulatory domain
ROCK	Rho-associated coiled-coil-forming protein kinase
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SM	sphingomyelin
SNS	sympathetic nervous system
TGN	*trans*-Golgi network
TNF	tumour necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis inducing ligand

INTRODUCTION

In 1982 it was discovered that tumour promoting phorbol esters activate members of the protein kinase C (PKC) family [1]. Since then, a myriad of studies have investigated the involvement of PKC signalling in carcinogenesis. The PKC family encompasses at least ten different isoforms, with similar requirements for activation, which makes it difficult to determine the specific cellular function of each protein. Thus, an increased knowledge of how the different PKC isoforms mediate their actions may be of great importance for the development of specific therapies that target the enzyme. The general aim of this thesis was to investigate if altered levels of PKC isoforms influences the cell death of malignant cell-lines. Furthermore, we wished to clarify how the localisation of PKC influences the biological activities of the enzyme, with emphasis on programmed cell death and neurite outgrowth.

In the following section I will review different aspects of molecular biology, which are important for the understanding of my research studies. I will discuss the family of PKC and their mechanisms of regulation and I will focus especially on the C1 domain, which has a central role in my studies. Next, I will introduce the Golgi apparatus, an organelle that I have come to know as an important site of PKC localisation. The Golgi apparatus is a vital place for lipid production and an intracellular pool of diacylglycerol (DAG), which is an activator of PKC. I will describe the interconnections between DAG and ceramide, which is another lipid that conceivably modulates the activity of PKC. Furthermore, I will briefly review the field of programmed cell death as well as the molecular basis of neurite outgrowth. Within these sections I will try to integrate the current knowledge of how PKC influences the processes above. Since, my experimental work concerning PKC mainly has been performed using neuroblastoma and prostate cancer cells as model systems I will eventually discuss the properties of cancers in general and neuroblastoma and prostate malignancies in particular.

BACKGROUND

The PKC family

In 1977, Nishizukas group discovered an enzyme that eventually was named PKC [2]. Later PKC was found to comprise a family of phospholipid-dependent serine/threonine kinases, consisting of at least ten different isoforms. These are divided into three subgroups based on their structure and different requirements for activation (Fig. 1). The classical PKCs (cPKCs; α, βI, βII and γ) are diacylglycerol (DAG) sensitive and require $Ca^{2+}$for their activation. The novel PKCs (nPKCs; δ, ε, η and θ) are activated by DAG but are insensitive to Ca^{2+}, while the atypical PKCs (aPKCs; ς and ι/λ) are insensitive both to Ca^{2+} and DAG. Phorbol esters mimic the actions of DAG and are often used in cellular experiments. All PKC isoforms are dependent on the acidic membrane lipid phosphatidylserine for their activation. Each isoform is encoded by a separate gene with the exception of PKCβI and PKCβII, which are splice variants of the same gene [3-5].

PKCμ and PKCν are human homologues of the protein kinase D (PKD) variants PKD1 and PKD3, which are found in mice [6]. Members of the PKD family are homologous to the PKCs within their C1 domains, while their catalytic domains are similar to the calcium/calmodulin dependent protein kinases. Hence they are classified as a subgroup within this family [6]

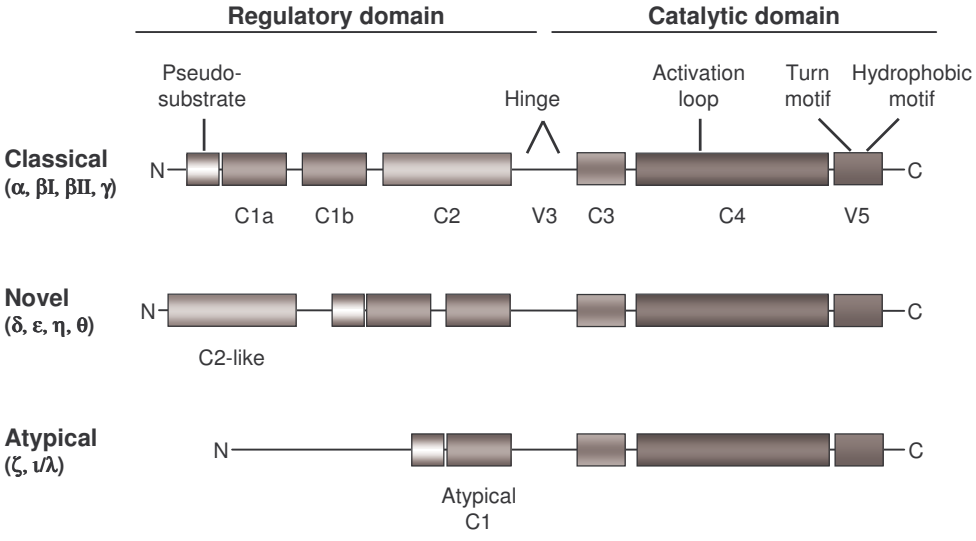

Figure 1. *Overview of the domain structure of the PKC family. Adapted, with permission, from Trollér, U. (2003). PhD thesis, Division of Molecular Medicine, Lund University, Sweden.*

Structure and function

The PKC molecule is a single polypeptide chain with a molecular weight of 77-87kDa, consisting of an N-terminal regulatory domain (RD) and a C-terminal catalytic domain (CD). This structure can be further subdivided into four highly conserved regions (C1-C4), which are separated by five variable regions (V1-V5) [3]. The characteristics of the different PKC domains are further discussed in the following sections.

Regulatory domain

Pseudosubstrate

The pseudosubstrate is an autoinhibitory region within the PKC molecule that is tethered to the catalytic core in the inactive enzyme. It contains the sequence of an optimal PKC substrate except for a serine that is substituted for an alanine in at the phosphorable position [7]. Once PKC is activated the pseudosubstrate is released, and since it is rich in basic residues it binds to acidic lipids in the plasma membrane and thereby stabilises the active conformation of PKC [8].

C1 domain

The C1 domain is cystein rich region of about 50 amino acids that initially was described as a binding site for DAG and phorbol esters [9]. Classic and novel PKCs have a tandem repeat of C1 domains designated C1a and C1b, while atypical PKCs have a single copy. C1 domains are divided into two groups; typical C1 domains, which bind phorbol esters and atypical C1 domains which do not [10]. In addition to classical and novel PKCs, typical C1 domains are found in PKD; chimaerins; Munc-13; Ras GRPs and DAG kinase β and γ. The atypical C1 domains, on the other hand, are found in atypical PKCs; vav; Raf-1; and the remaining seven DAG kinases [10-12].

The amino acid sequence of C1 domains is highly conserved with the consensus motif HX11-12CX2CX12-14CX2CX4HX2CX6-7C (H=histidine, C=Cystein, X= any amino acid residue), where the two histidines and six of the cysteins together coordinate two Zn^{2+} ions in each C1 domain [13, 15]. Thus, mutation of any of these essential residues alters the basic structure of the domain and affects the ability of ligand binding [13].

The three-dimensional structure of the C1 domain consists of two β-sheets and a C-terminal α-helix. The β-sheets form a hydrophilic groove that is surrounded by hydrophobic residues. Binding of DAG or TPA caps the hydrophilic cleft and generates a contiguously hydrophobic surface, which enables the domain to penetrate membranes [14, 15].

The consensus residues 8, 13, 20, 22 and 24 form the hydrophobic wall around the DAG-binding pocket that presumably is inserted in the membrane [10, 15]. These amino acids are always large and hydrophobic (Met, Val, Leu, Ile, Phe, Tyr or Trp) in typical C1 domains [10]. In atypical C1 domains they are often polar, which might explain their lack of ability to bind phorbol esters [10]. In accordance with this, Arg-20 in PKCζ has been suggested to contribute to the enzymes inability to bind phorbol esters [16]. On the surface of the C1

domain there is a belt of basic amino acids that interacts with phospholipid head groups [17].

Numerous studies show that the different C1 domains within PKC have inverse binding affinities for DAG/phorbol esters. The C1a domains of PKCα, PKCδ and PKCθ have low affinity for TPA, and the two former preferably bind DAG [18, 19, 20]. In contrast, the C1b domain of PKCα and PKCδ selectively respond to TPA [18, 21, 22]. It was recently suggested that C1 domains are unequally exposed and that the most accessible domain mediate the translocation of the enzyme. Although this seems to be the case for PKCδ, both C1 domains of PKCγ and PKCε bind DAG with high affinity and are mutually involved in membrane binding, since they are not conformationally restricted [18, 20, 23, 24].
In addition to DAG/TPA, C1 domains bind retinoic acid (RA)[25, 26] and possibly ceramide and arachidonic acid [28], which results in translocation of the proteins. The regulation of PKC by ceramide will be further discussed below.
Moreover, C1 domains mediate protein-protein interactions. Treatment with TPA induces translocation of β2-chimaerin to the Golgi where it, via its C1 domain, interacts with the transmembrane protein Tmp21-I [29]. Furthermore, the C1a domain of PKCβII interacts with the centrosomal protein pericentrin, and this association is important for the organisation of the microtubule [30].

C2 domain

The C2 domain was originally defined as the Ca^{2+} -binding site of cPKCs, but was later found to be present also in the Ca^{2+}-independent nPKCs as well as in several other proteins. The primary structure of the C2 domain consists of 130 amino acids that form a pocket consisting of eight antiparallel β-strands [31]. In the cPKCs, this pocket is lined with five aspartic acid residues that coordinate two or three Ca^{2+}-ions. However, in novel C2 domains, these residues are missing and the domain is unable to bind Ca^{2+} [32]. Binding of Ca^{2+} ions to the C2 domains of cPKCs increases the enzymes affinity for anionic membranes since the positively charged Ca^{2+}-ions neutralise the negative charge of phospholipid head groups and forms a bridge between the C2 domain and PS [33]. C2 domains may also act as membrane-targeting motifs through their protein-protein interactions with the RACKs (receptors for activated c-kinase) [34]

Variable regions

The five variable regions (V1-V5) of PKC are segments with low sequence homology, which separate the highly conserved C1-C4 domains. The V3 region forms a hinge between the regulatory and catalytic domain of PKC and is a site for caspase and calpain mediated proteolysis, which generates a constitutively active kinase domain [35-37]. This cleavage may be of importance during apoptosis, which is further discussed below.
The V5 region resides in the very C-terminus of PKC and consists of approximately 50

amino acids. The PKCβI and PKCβII isoforms differ only in their V5 region, and hence this region is likely mediating the differences in localisation between the isoforms observed in response to TPA [38, 39]. The V5 region of PKCβII has been reported to contain parts of the specific RACK1 binding site and thereby it determines the specific subcellular anchorage of the enzyme [40]. Moreover, the V5 regions of PKCδ and PKCε may mediate specific subcellular localisation and biological functions of the isoforms [41].

Catalytic domain

The catalytic domain of PKC consists of the ATP-binding C3 region and the substrate binding C4 region. Most PKC isoforms preferably phosphorylate substrates with serines and threonines surrounded by basic residues, probably since key residues in the substrate binding cleft are acidic [42]. The primary sequences of the novel and classical PKC family are 60% homologous within the catalytic domain. Moreover, the crystal structure of the catalytic domain of PKCθ is very similar to the crystal structures of protein kinase B (PKB)/Akt and protein kinase A (PKA) [43]. Thus, the specificity of PKC isoforms is believed to be dependent on its localisation next to substrates rather than actual substrate specificity [44].

Regulation of PKC

Phosphorylation

The biological function of PKC is dependent on a series of ordered phosphorylations. The first and rate-limiting phosphorylation is mediated by PDK-1 (phosphoinositide-dependent kinase-1) and occurs in the activation loop of the C4 domain of PKC. It has been suggested that both PDK-1 and PKC need to be recruited to the membrane by phosphoinositol-3,4,5-trisphosphate (PIP_3) and DAG, respectively, for this phosphorylation to occur [45]. Phosphorylation at the activation loop initiates subsequent phosphorylations of the turn motif and the hydrophobic sites, both located in the V5 region. In cPKCs these reactions are believed to occur through autophosphorylation, whereas a kinase complex including PKCζ may mediate phosphorylation of the hydrobhobic site of PKCδ [46]. The phosphorylation reactions lock PKC in a stable conformation that is resistant to proteases and phosphatases [32].

Membrane targeting and activation

Although the phosphorylated PKC is catalytically “mature”, it resides in the cytosol in an inactive conformation with the pseudosubstrate bound to the active site. Several groups have investigated the temporal and spatial activation and membrane targeting of PKC. In PKCα, the C1a domain and the C2 domain are tethered to each other via hydrogen bonds [47]. Stimulation of receptors trigger the release of Ca^{2+}, which binds to the C2 domain of PKCα and increases the enzyme's affinity of for anionic membrane lipids [33]. The interactions

with phosphatidylserine induces a conformational change of PKCα, whereby the C1a domain can be inserted into the membrane and bind DAG [47, 48]. It has been proposed that Ca^{2+} and DAG work synergistically since DAG increases the specificity of cPKCs for acidic phospholipids [49]. The association to phosphatidylserine is also essential for the activation of PKCδ, since it mediates the release of the C1a domain and consequently makes it accessible for DAG binding [20]. Since the C1 domains of PKCε are less conformationally restricted, phosphatidylserine may not be required for its binding to DAG [23]. Binding of DAG, in turn, induces a conformational change of PKC, which releases the pseudosubstrate from the active site [5, 27, 48]. Upon activation, PKC becomes ubiquitinated, which triggers its subsequent downregulation via the ubiquitin-proteasome pathway [50].

Anchoring

Several classes of anchoring proteins associate with PKC and mediate the subcellular localisation of the enzyme. One such family are the previously mentioned RACKs, which position active PKC isoforms and thereby bring them in close proximity with their substrates [34]. So far, two RACKs have been identified; RACK1 that binds PKCβII [51] and RACK2/β′COP that binds PKCε [52]. RACKs may also function as adaptor proteins that bind the C2 domains of several signalling enzymes and integrate their signals [34]. Mochly-Rosen and co-workers suggest that activation of PKC induces a conformational change that exposes a RACK binding site within the PKC molecule [44]. In the inactive PKC, this RACK-binding site is tethered to an intramolecular sequence, which resembles the sequence of the corresponding RACK (termed pseudo-RACK or ψRACK). Based on this theory, they have designed peptides, which specifically agonise or antagonise the translocation of PKCδ and PKCε and thereby regulate their function [53-57]. The translocation activators are obtained from the ψRACK sequence and are supposed to destabilise the closed conformation of PKC and hence increase its binding to the true RACKs. The translocation inhibitors, on the other hand, correspond to the RACK-binding sites of PKC and competitively bind the cellular RACKs [53-57].

The Golgi Apparatus

In addition to the plasma membrane, PKC localises to the cytoskeleton and intracellular membranes of different organelles [38]. One such subcellular compartment is the Golgi apparatus, which is discussed in the following section.

Structure and function

Camillo Golgi discovered the Golgi apparatus in 1898, but it was a controversy for more than 50 years whether the Golgi apparatus was a true organelle or just an artefact [58]. The Golgi apparatus, or Golgi complex, has a central role in the secretory pathway and it is the

site where newly synthesised proteins from the endoplasmic reticulum (ER) are modified and sorted for retention or further intracellular delivery. Furthermore, sphingomyelin and glycolipids are generated at the Golgi complex [59].
The Golgi complex is located at the cell centre next to the centrosome and consists of four to six separate sub compartments called stacks or cisternae. The Golgi complex is polarised both in morphology and function. Newly synthesised proteins enter the stack closest to the ER, called the *cis*-Golgi, and exit through the *trans*-Golgi network (TGN). During their transport through Golgi, the proteins are modified (e.g. glycosylated) and sorted and these processes take place in distinct regions of Golgi [59]. It has been debated for a long time how cargo proteins move through the Golgi apparatus. Some people argue that Golgi stacks containing the cargo proteins are formed on the *cis* face and gradually mature through a retrograde delivery of Golgi enzymes. Others propose that each stack has a distinct composition and that the cargo is delivered between the stacks by vesicular transport [60-62].
Independent of the preferred model, the small GTPases Sar1 and Arf1 regulate the transport of proteins between ER and Golgi, and within the Golgi complex, respectively [59, 62]. Newly synthesised proteins that are destined for the secretory pathway are targeted to specialised membrane domains of the ER. Active SarI then mediates the recruitment of COPII coating proteins and additional effector proteins, which are required for vesicle formation /maturation of the membrane compartment. Conversely, the small GTPase Arf1 regulates the assembly of COPI coating proteins, and mediates the retrograde transport of proteins from Golgi to ER [59, 62].

C1-domain containing proteins at the Golgi complex

Stimulation with DAG or its analogues induces translocation of several cytosolic C1 domain-containing proteins to the Golgi complex. One example is PKD that is targeted to Golgi via its C1a domain and regulates the fission of transport carriers from the TGN [63]. Studies from Malhotras group indicate that proline 155 in the C1a domain of PKD creates a binding site for DAG, and reduction of the DAG levels inhibits the recruitment of PKD to the TGN [63, 64]. Treatment with TPA promotes the association of β2-chimaerin with the *cis*-Golgi protein Tmp-1 and this is mediated by the C1 domain of β2-chimaerin [29]. Furthermore, the C1 domain containing protein RasGRP1 translocates to Golgi in response to TPA, where it activates Ras [65]. Munc-13 is translocated to the Golgi apparatus after treatment with TPA in opossum kidney cells. In light of our findings it is interesting that a deletion mutant of Munc-13, lacking the C1 domain, is unable both to localise to the Golgi and induce apoptosis [66].
Several PKC isoforms are targeted to the Golgi complex and have been implicated in membrane trafficking and Golgi function [67-69]. For instance, a Golgi-associated PKC has been proposed to regulate phospholipase D (PLD) mediated vesicle transport from the TGN [70]. Further work by Exton's group shows that PKCα translocates to the perinuclear region and

activates PLD via protein-protein interactions [71-73]. Activation of PKC stimulates vesicle transport by the recruitment of Arf1 and β′-COP to the Golgi membranes [74]. PKCε associates with the COPI coat protein β′-COP (also referred to as RACK2) and may thus be involved in this process [52]. PKCε has also been shown to localise to the Golgi via its C1 domain and regulate the sulfatation and secretion of glycosaminoglycans [75].
Finally, we and others show that PKCθ is targeted to the Golgi via its C1b domain [76, 152], but its interaction partners and mechanisms of action at the Golgi are so far unidentified. In this context it is interesting that phosphorylation of PKCθ at the activation loop unables its localisation to the Golgi [77].

Lipid turnover at the Golgi complex

The Golgi complex is an important site of lipid synthesis and the lipid composition of its membranes is crucial for the regulation of protein trafficking [78]. DAG and ceramide are two vital lipids present in the Golgi and their pathways of generation and clearance are highly connected (Fig. 2). Ceramide is generated through *de novo* synthesis by ceramide synthase in the ER and then translocates to the Golgi apparatus where it serves as a precursor for sphingolipids [79, 80]. The majority of the intracellular ceramide is however produced by sphingomyelinases (SMases), which hydrolyse sphingomyelin (SM) to ceramide and phosphocholine [81, 82]. Conversely, ceramide-signalling is attenuated by glucosylceramide synthase that incorporates it into glucosylceramide or through clearance mediated by cera-

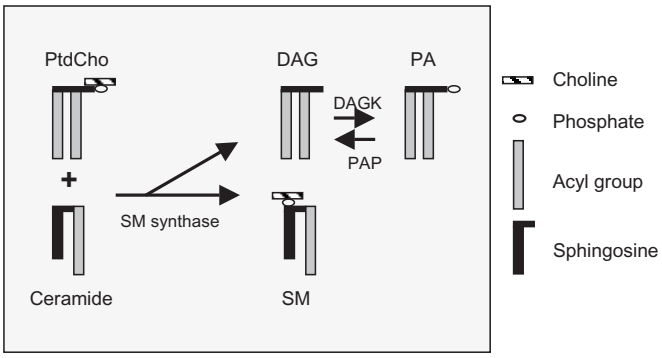

Figure 2. Lipid turnover at the Golgi. Ceramide is produced at the ER and transported to the Golgi complex. Sphingomyelin (SM) synthase converts ceramide and phosphatidylcholine (PtdCho) to DAG and SM. Phosphorylation of DAG by DAG kinase (DAGK) generates phosphatidic acid (PA). Conversely, DAG can be generated by dephosphorylation of PA by PA phosphatase (PAP).

midases. The majority of the DAG is generated through dephosphorylation of phosphatidic acid (PA, via PA phosphatase). DAG is however also produced from phosphatidylcholine during SM synthesis and by hydrolysis of phosphoinositol-4, 5-bisphosphate (PIP_2). Since DAG is a "bi-product" in the generation of SM from ceramide and phosphatidylcholine, one can reduce the cellular levels of DAG by inhibiting ceramide synthase (e.g. treatment with fumosin B1) [64, 83]. DAG is cleared through its phosphorylation to PA (via DAG kinase) and through synthesis of phosphatidylcholine [84].

Ceramide

Ceramide and DAG are similar in structure, but in contrast to DAG, the role of ceramide as a second messenger is controversial. The major criticism arises from the fact that the short chain ceramides used in many experiments have different biophysical properties and distribution compared to the cellular ceramide [85]. Ceramide is implicated as a second messenger in apoptotic processes, which is further discussed below. However, it has been proposed that ceramide exerts its apoptotic effects by altering the structure and fluidisation cellular membranes, diffusing to, and affecting, the mitochondrion and by disturbing the budding of vesicles [85]. Ceramide may also act proapoptotically by aggregating into membrane rafts and cluster death receptors [86].

PKC and ceramide

A consensus motif for ceramide binding has not been identified. Based on the structural similarities between DAG and ceramide, it was hypothesised that ceramide binds to C1 domains [87]. The C1b domains of PKCδ and PKCε have been shown to be important for ceramide induced translocation of the isoforms to Golgi [28, 88]. Moreover, our results demonstrate that Asp-257 together with Met-278 in PKCεC1b is essential for the modulation of PKCε functions by ceramide [89]. However, in the case for PKCα, the C2 domain is required for ceramide-induced translocation to the Golgi [90]. In this context it is interesting that ceramide does not bind the C1 domain of Raf-1, but the catalytic domain [91].
Ceramide has been suggested to function as a regulator of PKC activity. One of the primary candidate-targets is PKCζ that associates with, and is activated by, ceramide *in vitro* [92-94]. Ceramide suppresses the autophosphorylation of PKCδ in renal mesangial cells, suggesting an inhibitory role for ceramide on PKCδ activity [95]. In contrast, ceramide analogues selectively activate and translocate PKCα in these cells [95]. However, exogenously applied ceramide indirectly inhibits the kinase activity of PKCα in Molt-4 cells [96]. A recent report shows that endogenously produced ceramide inhibits the catalytic activity of PKCα and PKCθ in Jurkat cells [97]. Thus, the effect of ceramide on PKC may be cell specific. Ceramide is also implicated in PKC-mediated apoptosis in several cell systems, which is discussed further below.

Apoptosis

Apoptosis was first described in 1972 by Kerr and colleagues and the concept defines a series of cellular events that eventually lead to the death and removal of unwanted cells [98]. Apoptosis is characterised by cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. Through these actions the cell is neatly packaged and ready to be engulfed by neighbouring cells. Thus, no inflammatory response is induced by apoptosis, which contrasts the necrotic cell death where cells swell and the plasma membrane breaks. The balance between cell proliferation and cell death is important for normal tissue homeostasis and malfunctioning apoptotic responses are common in tumours [99, 100].

Caspases

The caspases comprise a family of proteases, which play a central role in the apoptotic response. As the name indicates, the caspases have a cysteine in their active site and cleave their substrates after specific aspartate residues (cysteine-dependent-aspartate-specific proteases). There are 14 mammalian caspases and while some of them are involved in apoptosis, others regulate inflammation by processing cytokines. The apoptosis-inducing caspases are divided into two groups: the initiator caspases (caspase -2, -8, -9 and - 10) and the effector caspases (caspases -3, -6, -7) [101, 102]. The members of both groups consist of a prodomain, a large subunit of 20 kDa (p20) and a small subunit of 10 kDa (p10). All caspases reside as inactive pro-caspases in the healthy cell, and they are proteolytically activated upon apoptosis. The initiator caspases are autoactivated in a process that requires them to associate to complexes of specific coactivators. Once activated, the initiator caspases process the effector caspases by removing the prodomain and releasing the p10 and p20 subunits, which later associates to a heterodimer. Two caspase heterodimers form a tetramer, which is the active form of the caspase [100, 101, 103-105].

Apoptotic pathways

Apoptosis can be initiated by various internal and external signals. There are two major apoptotic pathways: the intrinsic pathway and the extrinsic pathway. Which of these pathways that is activated depends on the origin of the apoptotic stimuli. In addition, organelles such as the ER, the nucleus, lysosomes and the Golgi complex (further discussed below) may be local sensors of cell-stress and initiate death signalling pathways [106].

The intrinsic pathway

The intrinsic pathway is also referred to as the mitochondrial pathway since it operates at this organelle. Cellular stresses such as chemicals and irradiation induce mitochondrial membrane permeabilisation (MMP) and the release of cytochrome *c*, which binds to apoptotic protease activating factor-1 (Apaf-1). Binding of cytochrome *c* enables Apaf-1 to recruit pro-caspase-9 molecules, which are activated by autoproteolysis. Active caspase-9 remains bound to Apaf-1, which is believed to function as a regulatory subunit of the enzyme [99, 101, 105, 107].

The extrinsic pathway

The extrinsic pathway is initiated by activation of cell surface death receptors such as Fas, tumour necrosis factor α (TNFα) and TRAIL (TNF-related apoptosis inducing ligand). Binding of a ligand induces receptor oligomerisation and the subsequent recruitment of other proteins that, in resemblance to the receptor, contain so called death domains (DD). In the case of CD95/Fas, one such DD containing adaptor protein is FADD (Fas-associated death domain), which together with pro-caspase 8, forms a protein complex called DISC (death-inducing signalling complex). Oligomerised pro-caspase-8 is autoproteolytically activated and subsequently released into the cytoplasm [108].

Execution of apoptosis

The two pathways often converge at the level of effector caspase activation. However, in some cell types, the main function of caspase -8 is to proteolytically activate the Bcl-2 family member Bid (se below). Bid promotes MMP and hence acts on the mitochondrial pathway. Eventually the apoptotic MMP leads to failure of the metabolic and redox functions as well as loss of mitochondrial inner transmembrane potential, which contributes to cell death [109].

The activated effector caspases initiate the degradation of the cells by cleavage of substrates such as nuclear laminins, cytoskeletal proteins and caspase-activated DNAses. The activation or inactivation of these proteins eventually leads to apoptotic characteristics such as nuclear shrinkage, loss of cell shape and DNA laddering, [100, 108].

The Bcl-2 family

The B cell lymphoma-2 (Bcl-2) family of proteins are another set of important apoptosis regulators. This family include both pro-and anti-apoptotic members. They are divided into three groups based on their function and homology within Bcl-2 homology (BH) regions. The first group consists of the antiapoptotic proteins. The most well characterised members are Bcl-2 and Bcl-XL, which contain four conserved BH domains (BH1-BH4) and a C-terminal transmembrane region. The second group contains proapoptotic proteins such as Bax and Bak, which are structurally very similar to Bcl-2 but lack the BH4 domain. The third group also contains proapoptotic proteins such as Bid, Bik, Bim and Bad. Since these proteins show homology only in the BH3 domain they are referred to as the BH3-only proteins [105, 110].

Although it is accepted that the Bcl-2 family influences apoptosis, the underlying mechanisms are still unclear. Bax-like proteins are critical for apoptosis and mediate a direct effect. In response to apoptotic signals, Bax-like proteins form homo-oligomers that interact with the mitochondria and induce the release of cytochrome *c* from the inner membrane. This may occur through a direct pore formation or interactions with pre-existing channels, such as the mitochondrial permeability transition pore. Bcl-2 can block these events through hetero-dimerisation with Bax and thus the balance between pro-and antiapoptotic Bcl-2 members is important for the outcome of an apoptotic signal. Most BH3-only proteins cannot directly

induce apoptosis, but bind to Bcl-2 and neutralise its antiapoptotic actions. The BH3-only member Bid, which is activated through caspase-8 mediated proteolysis, is an exception. In addition to Bcl-2 binding, Bid is also believed to trigger homo-oligomerisation of Bax and Bak and thereby activate them [105, 108, 110, 111].

Other regulators of apoptosis

In addition to the Bcl-2 proteins, there are other intracellular regulators of apoptosis. The inhibitors of apoptosis (IAPs) comprise a family of proteins that bind caspases and block their interactions with substrates. Furthermore, the SMAC/Diablo (second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI) proteins are released from the mitochondria simultaneously with cytochrome *c* and in turn inhibit the IAPs [100].

Golgi and apoptosis

Although the mitochondrion plays a central role in the integration of apoptotic pathways, other organelles such as the ER, lysosomes and the Golgi apparatus have also been suggested to sense cellular damage and activate local death pathways [106]. The Golgi harbours Fas and TRAIL receptors and activation of p53 induces a redistribution of Fas from Golgi to the plasma membrane, which make the cells sensitive to apoptosis [112, 113]. Moreover, induction of apoptosis by Fas leads to generation of GD3 gangliosides, which shuttle to the mitochondria and disrupts the MMP [114]. Both the cystein protease calpain II [115] and caspase-2 reside in the Golgi [116]. Caspase-2 cleaves the Golgi protein golgin-160 during staurosporine-induced apoptosis and prevention of this cleavage delays the disassembly of the Golgi. Furthermore, cleavage by caspase-2 induces nuclear accumulation of Golgin-160 fragments possibly by unmasking a nuclear localisation sequence [117]. In a recent review Machamer suggests that caspase-2 could initiate stress-induced apoptosis by proteolysis of Golgi proteins. The cleaved Golgi proteins would then induce apoptosis per se rather than just being the outcome of the apoptotic process [118].

Ceramide and apoptosis

Ceramide production is a universal phenomenon in cells undergoing apoptosis [119] and various apoptosis-inducing stimuli such as Fas ligand [120], γ-irradiation [121] and chemotherapeutics [122, 123] increases the endogenous levels of ceramide.

The mitochondrion is a central player in the apoptotic response and ceramide can induce mitochondrial disturbances by several means. Endogenously produced ceramide inhibits the mitochondrial respiratory chain and promotes generation of reactive oxygen species (ROS) at the mitochondria [124,125]. Moreover, cell permeable ceramide affects the mitochondrial permability transition pore [126] and ceramide-mediated activation of the aspartate protease cathepsin D results in cleavage of Bid and subsequent activation of caspase-9 and -3 [127].

PKC and apoptosis

PKC is involved in the regulation of apoptosis and the roles of the different isoforms in this process have received much attention in the literature. Individual PKC isoforms can be either pro- or antiapoptotic depending on the cell-type and the stimuli. In many of cell-systems PKCα and PKCε have emerged as antiapoptotic isoforms. Inhibition of PKCα induces apoptosis in both COS and salivary epithelial cells [128, 129] and PKCα phosphorylates Bcl-2 in myeloid leukaemia cells and thereby suppresses apoptosis [130]. PKCε induces Bcl-2 expression in myeloid cells [131] and it neutralises the proapoptotic protein Bax in prostate cancer cells [132]. Furthermore, PKCε inhibits apoptosis mediated by the intrinsic pathway in lung cancer cells [133]. Terrian and colleagues have suggested that PKCε is oncogenic in prostate cancer cells and claim that overexpression of PKCε is sufficient for rendering androgen-dependent LNCaP cells androgen-independent [134]. In addition to the neutralising effect on Bax, PKCε may mediate a constitutive activation of the ERK pathway, hyperphosphorylation of Rb and activation of the PKB/Akt survival pathway, [132, 134, 135]. The view of PKCε as a mediator of survival in androgen-sensitive prostate cancer cells was however recently challenged by Powell's group who suggest that it in fact is proapoptotic [136]

PKCδ appears to be mostly proapoptotic, which is discussed below. Also PKCθ may positively regulate apoptosis, for instance by upregulating the expression of FasL following T-cell receptor activation [137, 138]. However, the fact that PKCα induces apoptosis in prostate cancer cells [136, 139, 140], while PKCδ under some conditions has antiapoptotic effects (discussed further below) illustrates the lack of general roles for PKC isoforms. Many studies that deal with PKC-induced apoptosis suggest that it is regulated by proteolytical activation, translocation and tyrosine phosphorylation. These aspects will be discussed in the following sections.

Regulation of PKC in apoptosis

Proteolysis

In 1995, Emoto *et al* described that DNA-damaging agents induce proteolytical activation of PKCδ [35]. Since then, several reports suggest that treatment with apoptosis-inducing stimuli leads to cleavage of PKCδ, PKCε and PKCθ. PKCθ is cleaved by caspase-3 during Fas-induced apoptosis in Jurkat and myeloid leukaemia cells [141-143]. The cleavage has been suggested to attenuate PKCθ mediated survival signals by priming PKCθCD for proteasomal degradation in Jurkat cells [143]. However, overexpression of the isolated PKCθCD induces apoptosis in myeloid leukaemia cells [144]. PKCε is also proteolytically activated by stimulation of death receptors, probably via caspase-7 [141, 142, 145]. This activation has been attributed to the antiapoptotic function of PKCε [145].

Cleavage of PKCδ is well characterised and has been demonstrated in several cellular systems and with various apoptosis inducing stimuli. Some examples of this are treatment of salivary gland acinar or glioma cells by etoposide [146, 147], irradiation of myeloid leukaemia cells

[35], UV-exposure of keratinocytes [148] and activation of Fas [142]. Furthermore, PKCδ is cleaved in neutrophils undergoing spontaneous apoptosis [149, 150]. In some cellular systems, the cleavage of PKCδ is essential for an apoptotic response, and overexpression of full-length PKCδ has no lethal effect [36]. However, we and others have shown that both PKCδ- [151, 152] and PKCθ- [152] dependent induction of apoptosis can occur without cleavage of the isoforms.

Cleavage of PKCδ is mediated by caspase-3 and occurs in the V3 region. This generates a constitutively active catalytic fragment that, when overexpressed, induces apoptosis in the absence of additional stimuli [36, 142, 153, 154]. In contrast, cleavage of PKCδ and the generation of a free CD is important for the protective role of PKCδ during TRAIL-induced apoptosis [155]. Specific inhibitiors of PKCδ, as well as dominant negative mutants of PKCδ, inhibit both activation of caspase-3 and subsequent cleavage of PKCδ. This suggests that there is a positive feedback loop between PKCδ and caspase-3 [146, 147]. PKCδ mediated apoptosis can also be inhibited by expression of Bcl-2 or Bcl-XL [35, 151, 156]. We and others have shown that induction of apoptosis by PKCδ is independent of its kinase activity in neuroblastoma and aortic smooth muscle cells [152, 156].

Phosphorylation

Phosphorylation of tyrosine residues is an activation mechanism that is distinct for PKCδ and these residues are not conserved among the rest of the PKC family (for further details see [157, 158]). A diverse set of stimuli, such as treatment with TPA [159] and activation of PDGF [160] or IgE receptors [161, 162] induces tyrosine phosphorylation of PKCδ. This effect is believed to be mediated by members of the Src family of kinases. Moreover, various apoptotic stimuli induce tyrosine phosphorylation of PKCδ. Etoposide-induced cleavage of PKCδ by caspase-3 and subsequent induction of apoptosis requires phosphorylation of tyrosine residues in the regulatory domain of PKCδ [147]. Furthermore, treatment with ceramide, interferon-γ or H_2O_2 induces tyrosine phosphorylation of PKCδ, which is crucial for its proapoptotic effect [88, 163, 164]. In contrast, infection of glioma cells with Sindbis virus or stimulation of TRAIL, also induces tyrosine phosphorylation of PKCδ, which in this context is important for anti-apoptotic actions of PKCδ [155, 165].

Localisation

As previously discussed, PKC isoforms localise in distinct subcellular compartments and this positioning is important for their effects. Hence it is interesting that various apoptotic stimuli provoke different localisation of PKCδ. Reperfusion of ischemic rat heart cells induces translocation of PKCδ to the mitochondria, increases the levels of Bad and releases cytochrome *c* [166]. Moreover, treatment with TPA [167], H_2O_2 [168], chemotherapeutics, UV-irradiation and brefeldin A [169] induces targeting of PKCδ to the mitochondria with a subsequent release of cytochrome *c* and activation of caspase-3. In prostate cancer cells, ceramide activates and translocates PKCδ to the mitochondria and promotes caspase-9 activation, which in turn amplifies the production of ceramide [170]. Thus, PKCδ seems to

have a central role in mitochondrial-dependent apoptosis. The cell-type specific responses to a stimulus can be further illustrated by the fact that ceramide translocates PKCδ and PKCε from the plasma membrane to the cytosol in leukaemia cells [171], whereas it induces translocation of PKCδ to the Golgi in HeLa cells [88, 163]. In other systems, nuclear translocation of PKCδ is required for etoposide-induced apoptosis, and cleavage of PKCδ facilitates its nuclear import [147, 172]. In glioma cells, Sindbis virus infection and activation of TRAIL induces translocation of PKCδ to the ER, which is important for its antiapoptotic actions [155, 165].

Targets of PKCδ

As discussed above, PKCδ exerts some of its apoptotic effects by translocating to the mitochondria and modifying its functions. In addition, PKCδ has targets in the nucleus (Fig. 3). PKCδ constitutively associates with the non-receptor tyrosine kinase c-Abl [173], which similarly to PKCδ, is involved in the reactions to oxidative stress and DNA-damage [174]. In response to irradiation, c-Abl phosphorylates and activates PKCδ that subsequently translocates to the nucleus [173]. Conversely, oxidative stress induces activation of c-Abl mediated by PKCδ, which in turn leads to further activation of PKCδ by c-Abl [175]. Several other studies further indicate that there is a connection between the actions of c-Abl and PKCδ. Similarily to c-Abl, the catalytic domain of PKCδ regulates the function of the p53 homologue p73β [176, 177]. PKCδCD has been shown to associate with and phosphorylate p73β, which leads to its accumulation, activation and subsequent induction of apoptosis

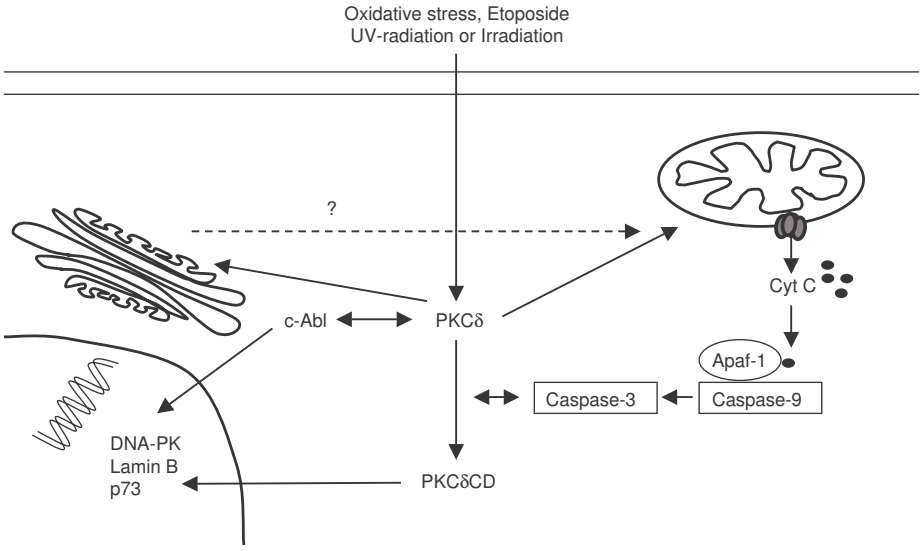

Figure 3. Regulation of cell apoptosis by PKCδ. See text for details.

[176]. Furthermore, PKCδCD interacts with and inactivates DNA-dependent protein kinase (DNA-PK), which is essential for the repair of double-stranded DNA breaks [153]. In this context it is interesting that c-Abl also phosphorylates and downregulates DNA-PK [178]. An early event in induction of apoptosis is exposure of phosphatidylserine on the surface of the plasma membrane. PKCδ may regulate this process by activating phospholipid scramblase that moves phosphatidylserine to the outer leaflet of the membrane [179]. Another apoptotic feature is the disassembly of the nuclear lamina and packaging of the nucleus into apoptotic bodies. In this process, PKCδ phosphorylates lamin B, which is required for its degradation by caspase-6 [180].

Furthermore PKCδ has been proposed to act upstreams of various members of the mitogen-activated protein (MAP) kinase superfamily. The p38 MAPK and c-Jun terminal kinase (JNK) cascades are generally associated with cellular stress, whereas the extracellular-signal-regulated kinases (ERKs) promote proliferation [181]. TPA induces apoptosis in prostate cancer cells through PKCα and PKCδ dependent activation of p38 MAPK [136, 182]. Moreover, in salivary parotid cells, etoposide induces sustained JNK activation and decreases ERK1/2 activity and PKCδ is required to mediate this effect [146]. PKCδ may also promote apoptosis by inducing dephosphorylation of Akt/PKB and thereby attenuating this survival pathway [166].

PKCδ in mouse-models

One approach to elucidate the specific functions of the PKC isoforms is the generation of knockout mice (KO) that lack expression of a particular PKC isoform. Generation of PKCδ KO mice revealed that these animals do not have an increased susceptibility to tumour formation [183]. However, they have an impaired clonal deletion of autoreactive B-cells, which leads to autoimmunity [183, 184]. Moreover, the PKCδ KO animals show an accumulation of smooth muscle cells, caused by a decrease in cell death, and hence develop arteriosclerosis [185]. Thus, these studies emphasise the importance of PKCδ in apoptosis-signalling.

Mochly-Rosen and co-workers have performed extensive studies of the roles of individual PKC isoforms during cardiac ischemia and reperfusion damage [53, 57, 186, 187]. They utilise transgenic mice that express the previously discussed PKCδ activator ψδRACK or the PKCε activator ψεRACK and demonstrate that while activation of PKCδ increases the damage of ischemia, PKCε act protective. Moreover, they show that isoform-selective peptides, which activates PKCε before ischemia, and inhibits PKCδ during reperfusion, protects the heart from damage [53, 57, 186, 187].

Anti-apoptotic effects of PKCδ and PKCθ

Although PKCδ generally is proapoptotic, this isoform may protect against cell death in some systems. Several studies have shown that activation of nPKCs protect cells from death receptor induced apoptosis by inhibiting DISC formation and activation of caspase-8 [141, 188-190]. Although these studies do not identify the particular isoform that mediates the antiapoptotic effect, others have shown that PKCδ mediates antiapoptotic effects of TNF-α

in neutrophils [191]. Recent results from Brodie and colleagues show that PKCδ protects glioma cells from TRAIL-induced apoptosis by increasing the phosphorylation of Akt/PKB [155]. The antiapoptotic effect of PKCδ in glioma cells is also observed in response to Sindbis virus infections [165]. Under both conditions, the cleavage of PKCδ and its translocation to the ER is important for the protective effect. PKCδ is also involved in the block of apoptosis induced by basic fibroblast growth factor in serum-deprived PC-12 cells [192] and protects non-small lung cancer cells from chemotherapy-induced apoptosis [193].
PKCθ is mainly expressed in hematopoietic tissue but is also found in muscle cells and in the embryonic nervous system [194, 195]. The functions of PKCθ have mostly been studied in T-cells, where it is important for T-cell receptor signalling, production of interleukin-2 and clonal expansion [196]. In addition, PKCθ mediates T-cell survival by phosphorylating, and thereby inactivating, the proapoptotic protein Bad during Fas-induced apoptosis [143, 197]. Conversely, T-cell receptor activation also leads to PKCθ mediated upregulation of FasL [137, 138]. Thus, it seems like PKCθ has a complex role in this pathway promoting either apoptosis or cell-survival.

NEURITE OUTGROWTH

Members of the PKC family regulate a multitude of cellular processes and are important for the control of the morphological changes of the differentiating neuron.
The undifferentiated neuron is spherical and lacks the polarised morphology of its mature counterpart. External factors such as extracellular matrix proteins, cell adhesion molecules (CAMs) and local concentration gradients of soluble molecules are believed to initiate intracellular signalling and the formation of membrane microdomains. These localised membrane domains are stabilised by additional membrane from the exocytotic pathway and are coated

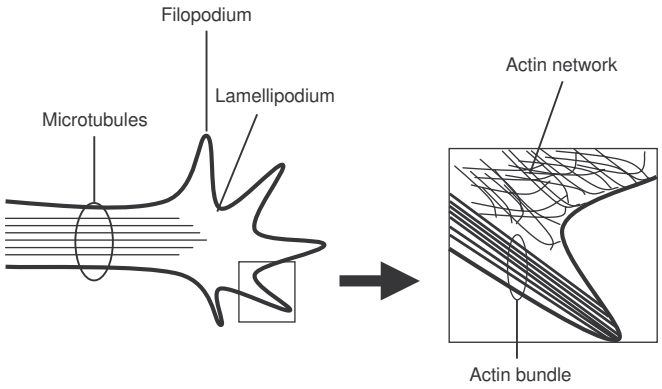

Figure 4. Growth cone at the distal tip of a neurite. The cytoskeleton of the neurite shaft is mainly composed of microtubuli, while the filopodia and lamellipodia consists of actin filaments. (Modified from Smith, 1988).

by filamentous actin (F-actin), which constricts the cell membrane and induces the formation of a bud. The bud then elongates to a process with a leading edge called the growth cone and eventually differentiates into an axon or a dendrite [198].
The edge of the growth cone is composed of two types of dynamic motile structures: long spikes called filopodia, which consists of actin bundles, and flat sheet like structures with a crosslinked meshwork of actin called lamellipodia. The more central part of the growth cone is mainly composed of hollow cylinders of parallel microtubuli, which facilitate fast intracellular transport and make the protrusion rigid (Fig. 4). Actin polymerisation occurs at the leading edge of the growth cone, which mediates extension of filopodia and lamellipodia. There is however also a simultaneous retrograde flow of actin from the leading edge, which mediates retraction. The net growth of a protrusion is therefore dependent on the rate of actin polymerisation and retrograde flow [199-201].

The Rho family of GTPases regulates the actin cytoskeleton

Rho A, Cdc42 and Rac are members of the Rho family of small GTPases and are important regulators of the actin cytoskeleton. Rho GTPases cycle between an active GTP bound state and an inactive GDP bound state. Guanine nucleotide exchange factors (GEFs) exchange GDP to GTP and hence function as activators, while GTPase-activating proteins (GAPs) increase the rate of GTP hydrolysis and function as inhibitors. The guanine nucleotide dissociation inhibitors (GDIs) prevent the dissociation of GDP from the inactive GTPase and thus also inhibit the proteins [202, 203]. Rho A is activated by extracellular cues such as lysophosphatidic acid, which induces the formation of stress fibers and focal adhesions in fibroblasts [204]. In contrast, Cdc42 promotes the formation of filopodia, while RacI causes the formation of lamellipodia [205, 206]. The actin cytoskeleton is essential for the regulation of the morphology of the neuron. In general Rho A suppresses neurite outgrowth. This is illustrated by the fact that C3 exoenzyme mediated inhibition of Rho A induces neurite

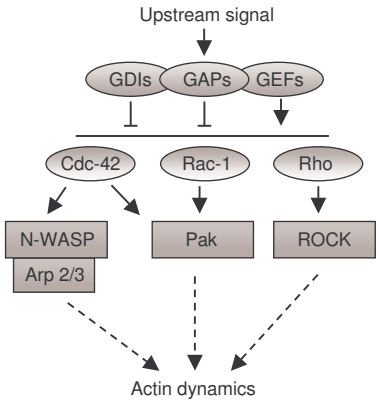

Figure 5. Rho GTPases regulate the actin dynamics. A simplified model showing the pathways that regulate actin dynamics via RhoA, Rac and Cdc 42.

outgrowth, while activation of Rho A induces neurite retraction [207, 208]. However, both Rac and Cdc42 are positive regulators of neurite outgrowth [199, 203, 209, 210].
The Rho GTPases regulate actin polymerisation through their downstream effector proteins (Fig. 6) [203]. Active Cdc42 recruits N-WASP and the actin-nucleating factor Arp2/3 that induces *de novo* actin polymerisation. Both Rac and Cdc42 also activate p21-activated kinases (PAKs), which in turn phosphorylate LIM kinase and inhibits the actin depolymerising protein ADF/cofilin [198, 199, 203]. In addition, the Rho GTPases regulate the myosin motor activity, which drives actin-based motility. The Rho A kinase ROCK (Rho-associated coiled-coil-forming protein kinase) is a well-studied Rho effector. ROCK positively regulates the motor activity of myosin II, which promotes bundling of actin filaments and generates contractile stress fibres. Active ROCK inhibits neurite outgrowth in neuroblastoma cells, an effect that is abrogated by the ROCK inhibitor Y-27632 [198, 211, 212].

PKC and neurite outgrowth

PKCε and PKCδ have emerged as important regulators of neurite outgrowth in neuronal cell lines. PKCε is enriched in growth cones of differentiating neuroblastoma [213] and PC12 cells [54] and several reports suggest that PKCε is a mediator of neurite outgrowth induced by NGF [54, 214, 215], RA [216, 217] and NCAM [218]. In addition, PKCδ has been suggested to positively regulate NGF-mediated neuritogenesis in PC-12 cells [219, 220]. Treatment with NGF increases the association of PKCε with the cytoskeleton [215]. Targeting of PKCε to the cortical cytoskeleton and/or the plasma membrane may be important for its neurite inducing capacity [217]. However, activation of PKCε may also induce collapse of growth cones [221].
Results from our group show that the neurite-inducing effect of PKCε is mediated by its regulatory domain and is hence independent on the catalytic activity [216]. In addition to neuroblastoma cells, this effect is also seen in the neuronal progenitor cell lines RN33B and HiB5 [207]. Moreover, deletion studies show that a small fragment encompassing four amino acids N-terminal and twenty amino acids C-terminal of the PKCεC1 domains is critical for neuritogenesis [222].
PKCθ has been implicated in the differentiation process of PC-12 cells [223] and LAN-5 neuroblastoma cells [224] and the expression levels of the isoform is increased during differentiation induced by NGF and RA. In addition, the cPKCs may be involved in neurite induction. PKCβII is implicated in NCAM-mediated neurite outgrowth [218, 225], while PKCα mediates neurite induction in hypothalamic neurons [226]. In contrast, myelin components may activate cPKC, which inhibits neurite outgrowth in primary neurons [227, 228].

Ceramide and neurite outgrowth

Ceramide has been suggested to act both as a positive and a negative regulator of neuronal cell differentiation. Treatment of neuroblastoma Neuro2a mouse cells with RA induces ceramide production leading to differentiation, and exogenously applied ceramide induces neurites

[229]. Furthermore, ceramide is generated in response to NGF treatment and stimulates the formation of neurites in hippocampal neurons [230, 231]. However, both endogenous ceramide and C_2-ceramide have been shown to inhibit neuritogenesis in a variety of neuronal cells [232-236]. There is no clearly identified mechanism behind the abrogated neurite outgrowth. Although some argue that exogenously applied ceramide increases the levels of glycosphingolipids/gangliosides, which in turn inhibits neurite outgrowth [235], others argue that ceramide itself exerts the effect [232]. Ceramide has also been suggested to mediate disruption of the actin network, block integrin-mediated cell adhesion and decrease the phosphorylation of the microtubuli polymerising protein Tau [234, 237, 238].

CANCER

General aspects

The transformation of normal cells to tumour cells is believed to occur through a multi-step accumulation of genetic changes. This process is well defined in a form of hereditary colon cancer, were an initial alteration in a gatekeeper gene induces a survival advantage of the cell and clonal expansion. Through a subsequent series of mutations the cells then evolve in a Darwinian manner [239]. Hanahan and Weinberg propose that the following six cellular alterations are common among most malignancies: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis and tissue invasion and metastasis [240]. The alterations occur in a small set of genes that are grouped as tumour suppressor genes, proto-oncogenes and stability genes [242]. The stability genes comprise a group of DNA-repair genes that minimise the number of mutations, and hence this group is important for keeping the genome stable [241, 242]. Tumour suppressor genes promote cancer when inactivated, which generally requires loss of function of both alleles. They are often regulators of growth inhibition, differentiation or apoptosis and can be exemplified by the well-characterised Rb (retinoblastoma) and p53 tumour suppressors. In contrast, proto-oncogenes induce tumours through gain-of-function (the altered form is referred to as an oncogene) and thus only require mutation in one allele. These mutations are often somatic and result in hyperactivity through overexpression or malfunction of the gene-product. Vogelstein and Kinzler suggest that all genetic alterations influence a small set of signalling pathways that are common among cancers [242]. Potential oncogenes occur at different levels in signalling cascades, e.g. the receptor tyrosin kinase (RTK) pathway. In this pathway, mutations can strike growth factors (e.g. PDGF), membrane receptors (e.g.. EGF-R/erbB, HER2/neu), as well as intracellular signal transducers (e.g. BCR-Abl, Ras, Raf) [242, 243].

Neuroblastoma

Neuroblastoma is the most common extracranial solid tumour affecting children and less than 50% of the patients survive the disease [244]. The tumours originate from immature cells of the developing sympathetic nervous system (SNS) and are most commonly located

in the adrenal medulla [245]. Apart from Schwann cells, the embryonic SNS consists of sympathetic neurons and small intensely fluorescent (SIF) cells, comprising the sympathetic ganglia, and neuroendocrine chromaffin cells that reside in the paraganglia and the adrenal medulla [246]. Neuroblastoma is a heterogeneous disease and while some tumours are utterly aggressive and resistant to therapy, others differentiate or regress spontaneously. The tumour prognosis is predicted based on the stage of the disease, the age of the patient at diagnosis and the site where the primary tumour is located. In general, infants that are less than one year have a good outcome, while a primary tumour of the adrenal gland is associated with a bad prognosis. In addition, genetic alterations can be used for clinical prognosis. Amplification of N-MYC, deletion of chromosome 1p and expression of TrkB is considered to be associated with a poor prognosis, while expression of TrkA is correlated with a good outcome. Low-stage tumours can be cured by surgery alone, whereas more aggressive neuroblastmas are treated with surgery combined with intensive chemotherapy and irradiation [244, 245, 247].

Cancer of the prostate

The normal prostate is composed of epithelial glands surrounded by fibromuscular stroma. There are three types of epithelial cells: basal, secretory luminal and neuroendocrine cells, and they are regulated in a paracrine manner by the stromal cells [248]. The growth and proliferation of normal prostate cells is regulated by androgen dependent secretion of growth factors. Secretion of LH-RH (luteinising hormone releasing hormone) from the hypothalamus induces release of gonadotrophins from the pituitary and subsequent secretion of testosterone from Leydig cells in the testes. In the prostate, testosterone is converted to 5α-DHT, which binds to androgen receptors (ARs) that promote transcription of target genes and thereby induces growth and survival of the cells [249]. Prostate cancer accounts for 33% of the male malignancies and is the second most common cause of cancer related death among men [250]. The tumours are treated with surgery or irradiation, but many patients fail this therapy and are thus subjected to androgen ablation therapy (i.e. high levels of LH-RH analogues that induce a down-regulation of the receptors in the pituitary and a reduced testosterone production). Many forms of prostate cancers are initially androgen dependent, but after 4-8 months of treatment, androgen-independent and highly malignant tumours frequently recur [249, 251]. This has been suggested to occur through an increased AR expression by gene amplification [252], mutations in the AR that decrease its specificity to different androgens [253] or an increased local production of androgens in the prostate [251]. Furthermore, the AR might be activated by ligand-independent mechanisms. Upregulation of growth factors (i.e. insulin-like growth factor 1 and epidermal growth factor) and/or RTKs such as HER-2/neu has been proposed to mediate indirect activation of AR through the Akt/PKB or MAPK pathways [249, 251]. In addition, prostate cancers often have reduced expression levels of the tumour suppressor phosphatase PTEN, which leads to an increased activity of the PKB/Akt survival pathway [251, 254, 255].

THE PRESENT INVESTIGATION

Aim

The general aim of this thesis work has been to investigate if altered levels of specific PKC isoforms influence the apoptotic responses in malignant cell-lines. Furthermore, we wished to elucidate how the localisation of PKC influences its biological activities. For this purpose, I have mainly used cell-lines derived from neuroblastoma and prostate cancer as model systems for my investigations.

The specific aims were:

- To identify proapoptotic PKC isoforms in neuroblastoma and prostate cancer cell-lines.
- To characterise the apoptotic mechanisms of these isoforms.
- To analyse the importance of specific PKC localisation for its ability to induce apoptosis and neurites.
- To characterise which subdomains and structures of the PKC molecule that are important for localisation to the Golgi complex.

Results and Discussion

Effects of PKC expression on neuroblastoma cell apoptosis- a role for PKCδ and PKCθ (Paper I and II).

Although PKC clearly is involved in apoptosis signalling, there are no general roles of different PKC isoforms in this process. Previous results from our group show that selective inhibition of cPKCs using Gö6976 induces apoptosis in neuroblastoma cells, while GF109203X, which inhibits both cPKCs and nPKCs, has moderate effects on apoptosis [256]. Moreover, previous data demonstrate that selective inhibition of PKCβI abrogates neuroblastoma cell proliferation [39]. Thus, cPKCs seem to be involved in neuroblastoma cell survival and proliferation.

In paper 1, we continue this work by investigating if individual nPKC isoforms have proapoptotic effects in neuroblastoma cells. For this purpose, we mainly use the malignant and multidrug resistant SK-N-BE(2) neuroblastoma cell-line. In the majority of our experiments we use expression vectors encoding PKC fused to enhanced green fluorescent protein (EGFP), which makes it easy to identify transfected cells expressing the fusion proteins.
As a first step, we investigate the effect of treatment with TPA and/or GF109203X on SK-N-BE(2) cells expressing PKCδ, PKCε or PKCθ. Apoptotic cells are identified by propidium iodide staining and cells are scored as apoptotic based on their nuclear morphology. TPA treatment of cells expressing PKCδ or PKCθ induces apoptosis, while cells expressing EGFP or PKCε are unaffected. Moreover, simultaneous treatment with GF109203X does not abolish the apoptotic response, but further augments the effect of TPA. GF109203X inhibits PKC competitively by interacting with the ATP binding site [257]. In a recent paper, our group found that treatment with GF109203X increases the DAG sensitivity of PKCα, conceivably by destabilising the closed conformation of the enzyme [258]. These results highlight the possibility that GF109203X may potentiate the apoptosis inducing effect of TPA by making the C1 domains more available for phorbol ester binding. Similar to a previous study in aortic smooth muscle cells [156], TPA only induces apoptosis in cells overexpressing PKCδ or PKCθ. We speculate that this effect could be caused by a simultaneous activation of other phorbol-ester sensitive proteins that counteract the apoptotic effects of endogenous PKC. This hypothesis is further supported by our previous study, showing that inhibition of cPKCs induces cell death in neuroblastoma cells [256].

In paper 2 we study the apoptotic response of neuroblastoma cells to treatment with ceramide, which has been suggested to be a modulator of PKC. Treatment with cell-permeable C_2-ceramide reduces the viability of SK-N-BE(2) and SH-SY5Y cells when quantified by MTT assays. This is in accordance with previous reports were C_2-ceramide induce cell death in SH-SY5Y and SK-N-SH neuroblastoma cells [259, 260]. Based on the fact that PKCδ is implicated in ceramide induced cell death [170, 171, 261], together with our previous finding

that expression of PKCδ or PKCθ is required for TPA-induced apoptosis in SK-N-BE(2) cells, we hypothesise that PKC-overexpression may augment the effect of C_2-ceramide. To probe specifically for apoptosis, we stain the cell nuclei with propidium iodide. This experiment reveals that C_2-ceramide induces apoptosis in cells overexpressing PKCδ or PKCθ, and that it has only moderate effects on PKCε-overexpressing cells. Moreover, the EGFP expressing control cells are unaffected by C_2-ceramide. Co-treatment with GF109203X only partially reduces the apoptotic effect, and this suggests that the regulatory domain of the isoforms mediates the effect to some extent. In conclusion, the results from paper 1 and 2 implicate that PKCδ and PKCθ are proapoptotic in neuroblastoma cells.

There seems to be an inconsistency between the data obtained with the MTT assay and the propidium iodide stainings regarding the levels of cell death in non-PKC transfected cells (paper 2). MTT assays measure the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide) into formazan and this process requires intact and metabolising mitochondria. Thus, MTT assays do not discriminate between a suppression of cell proliferation and an increase in cell death caused by necrosis or apoptosis. Furthermore, the MTT assay analyses both adherent and floating cells, whereas propidium iodide staining only detects the adherent cells that remains after washing and fixation. To further characterise the reduction in cell-viability, we treat SK-N-BE(2) cells with increasing concentrations of C_2-ceramide and look at the number of cells that detach from the dish and are floating in the medium (unpublished results). This experiment reveals that in C_2-ceramide-treated dishes, a significantly larger number of cells are floating and positive for trypan blue staining, indicating that they are dead. Hence, a conceivable explanation of our results is that in the absences of PKC overexpression, C_2-ceramide induces necrosis while the expression of PKC may shift the cell death towards apoptosis.

Expression of PKCα, PKCδ or PKCε renders androgen-independent prostate cancer cells sensitive to TPA-induced cell death (Paper IV).

The results above suggest that specific PKC isoforms are important for induction of cell death in neuroblastoma cells. In paper 4 we extend our studies and investigate how differences in PKC expression affect the susceptibility of prostate cancer cells to TPA-induced apoptosis. It is well documented that TPA treatment induces apoptosis in androgen-sensitive LNCaP cells, while androgen independent PC-3 cells are unaffected [262, 263].
We start out by investigating if overexpression of PKCα, PKCδ or PKCε can alter these responses. In accordance with previous studies, we demonstrate that PKCα and PKCδ potentiate the effect of TPA-treatment in LNCaP cells [139, 151, 182]. A similar but less pronounced effect of these isoforms is also seen in PC-3 cells after TPA-treatment, supporting the previous finding that PKCδ is important for apoptosis in this cell-line [170, 264]. In addition, we confirm the recent and somewhat controversial finding that PKCε augments TPA-induced apoptosis in androgen-dependent prostate cancer cells [136], and we show that

overexpression of PKCε also renders PC-3 cells sensitive to TPA. These results are in striking conflict with proposals from Terrian and colleagues who suggest that PKCε is oncogenic and protects prostate cancer cells from apoptosis [132, 134].
Treating cells with GF109203X blocks TPA induced cell death in PKC-overexpressing PC-3 cells, and a kinase-dead (KD) PKCε fusion protein is unable to induce apoptosis after TPA treatment. Thus, we conclude that PKCε is dependent on its kinase activity for induction of apoptosis in PC-3 cells. The results regarding LNCaP cells are however harder to interpret. Although GF109203X inhibits the apoptosis inducing effect of TPA in PKCα and PKCε overexpressing cells, it only partially abrogates cell death in PKCδ overexpressing cells.
However, overexpression of KD PKCα, PKCδ or PKCε fusion proteins does not suppress TPA-induced apoptosis in LNCaP cells, which contradicts previous reports by Kazanietz and coworkers [139, 151]. These results may be explained by the recent discovery that PKCα and PKCε have redundant roles in androgen-sensitive prostate cancer cells and must be knocked-down simultaneously for inhibition of TPA-induced apoptosis [136]. Furthermore, PKCδKD may induce apoptosis independent of its kinase activity, which is supported by our finding that GF109203X only to some extent inhibits the TPA-induced cell death.

Examination of endogenous PKC levels by quantifications of Western blots reveals that the level of PKCα is almost four-fold higher in PC-3 cells than in LNCaP cells. In contrast, the level of PKCδ is higher in LNCaP cells than in PC-3 cells. Moreover, we detect moderate levels of PKCε in both PC-3 and LNCaP cells with no differences in the expression levels. Taken together, our results so far support the theory that TPA-induced apoptosis of prostate cancer cells requires a combination of PKC isoforms. Furthermore, the fact that PKCε is expressed at similar levels in both cell-lines further contradicts the proposed ongogenic role of the protein.

Mechanisms of apoptosis induction by PKCδ and PKCθ in neuroblastoma cells (Paper I).

In the next set of experiments we wish to elucidate the mechanisms behind PKC-induced apoptosis in neuroblastoma cells. PKCδ and PKCθ are proteolytically activated in several cell-systems during apoptosis [142, 144, 147, 148, 265]. This cleavage is mediated by caspases and generates free catalytic (CDs) and regulatory domains (RDs).
To analyse whether the free CDs can induce apoptosis, we overexpress the isolated CDs of PKCδ, PKCε or PKCθ in SK-N-BE(2) cells. This experiment shows that both PKCδCD and PKCθCD induce apoptosis, whereas PKCεCD lacks a major effect. To confirm that the apoptotic effects are provoked by the kinase activity of PKC, cells are treated with GF109203X. Indeed, the inhibitor abrogates the induction of apoptosis. Thus, these results confirm the previous proposal that the catalytic domains of PKCδ and PKCθ are proapototic [36, 142, 144, 153, 154]. Since our first experiments suggest that the full-length (FL) isoforms of PKCδ and PKCθ induce apoptosis, independent of their catalytic activity, we next investigate if the

isolated RDs are able to mediate cell death. Overexpression of the RDs of PKCα, PKCβ, PKCδ, PKCε, PKCη or PKCθ show that all regulatory domains induce apoptosis to some extent, but the largest effect is observed in cells overexpressing PKCθRD. It should be noted that this effect is not caused by higher expression levels of PKCθRD compared to the other fusion proteins. We find it interesting that PKCθ can induce apoptosis, independent on its catalytic activity, and hence confirm this effect in two other neuroblastoma cells, KCN-69 and SK-N-SH. A similar effect is seen in vascular smooth muscle cells, were PKCδ induces apoptosis independently of its kinase activity [156]. To the best of our knowledge, there are however no previous reports showing an apoptotic effect of an isolated RD of PKC.

Based on the fact that FL PKCδ and PKCθ are apoptotic only after treatment with TPA, we hypothesise that TPA induces proteolytical activation of the enzymes. This would generate free CDs and RDs that could augment the apoptotic response. To test the hypothesis, we express PKCδ and PKCθ in SK-N-BE(2) cells, challenge the cells with different combinations of TPA and/or GF109203X and analyse the whole cell lysates by Western blotting. However, we cannot detect a cleavage of neither PKCδ nor PKCθ. This is in accordance with previous results from Kazanietz group, where FL PKCδ is intact after TPA induced apoptosis in LNCaP cells [151]. Moreover, our data suggest that the apoptotic effect of the isolated domains of PKCδ and PKCθ are mediated by a different mechanism than the TPA treated FL proteins. It is conceivable that TPA alters the localisation or conformation of PKC and thereby renders it apoptotic.
To further characterise the apoptotic effect of PKCδFL, PKCθFL, PKCδCD and PKCθRD, we treat transfected cells with an inhibitor of caspase-3. In addition, we perform cotransfection experiments with a vector encoding the antiapoptotic protein Bcl-2. Both caspase-3 inhibition and overexpression of Bcl-2 protects the cells from apoptosis. Thus, the intrinsic pathway of apoptosis may be involved in the apoptotic effects of these PKC fusion proteins.

Both the C1 and the C2 domains are required for PKCθRD-mediated apoptosis (Paper I).

Our next goal is to identify which parts of of PKCθRD that are essential for induction of apoptosis. For this purpose, we create a series of PKCθ derived constructs and examine their apoptosis-inducing capacity. Transfection experiments show that although the entire PKCθRD is proapoptotic, the other fusion proteins have minor impact on cell death. This is not dependent on a higher expression level, since both PKCθRD and PKCθPSC1V3 are expressed at lower levels than the other fusion proteins. This experiment clearly suggests that both the C1 and the C2 domains are required for induction of apoptosis by PKCθRD in neuroblastoma cells.

The C1 domain is crucial for Golgi-localisation of PKCθRD (Paper I and III).

When analysing the apoptotic effects of PKCθRD constructs we notice obvious differences in their localisation patterns. While PKCθC2, PKCθC2PS and PKCθC2PSC1a all localise throughout the cells, PKCθRD and PKCθPSC1V3 reside in a punctuate pattern in the perinuclear region. Since we previously have detected PKCθ only at mRNA level in SK-N-BE(2) cells [256], we use Jurkat cells to investigate the endogenous expression pattern of the isoform. Cells are treated with anti-Fas antibody for 6h, which induces cleavage of PKCθ [142], and are subjected to immunofluorescence staining with antibodies directed towards PKCθRD. Confocal images show that PKCθ initially localises throughout the cytoplasm, but after Fas-treatment the antibody is detected in a punctuate pattern. Since the antibody cannot discriminate between PKCθFL and the isolated RD, these results either indicate that the FL protein is redistributed upon induction of apoptosis or that the cleaved RD localises to these punctuate structures. Expression of the PKCθRD-EGFP fusion protein in Jurkat cells and examination by confocal microscopy reveals a similar punctuate localisation pattern.

The indication that PKCθRD may relocalise to perinuclear structures at induction of apoptosis implies that the localisation may be important for the effect. To identify this subcellular structure we transfect SK-N-BE(2) cells with PKCθRD-EGFP and use a series of markers for different organelles. Confocal images show a clear co-localisation of PKCθRD-EGFP with syntaxin 6 and 58K, which are markers for the TGN and the *cis*-Golgi, respectively. However, experiments with TPA-treated SK-N-BE(2)C cells expressing PKCθFL-EGFP reveal that this protein does not localise to the TGN. Thus, Golgi localisation is not important for the apoptotic effects of PKCθFL, which confirms our previous results suggesting that PKCθFL and PKCθRD exert their apoptotic effects via different mechanisms in neuroblastoma cells.

Identification of an amino acid residue in the PKC C1b domain that is crucial for its Golgi localisation (Paper I and III).

PKCθRD and PKCθPSC1V3 both localise in the punctuate pattern identified as the Golgi complex, whereas the other PKCθRD fusion proteins localise throughout the cell. These results indicate that the C1b domain is important for the Golgi localisation.
To further investigate this, we make a new set of PKCθ constructs encoding the tandem repeat of C1 domains (i.e. C1a + C1b) or the isolated C1a and C1b domain, all fused to EGFP. Confocal images of transfected cells stained with antibodies towards syntaxin 6 confirm that the majority of the C1b domain containing proteins localise to the TGN, while the isolated C1a domain resides in the cytoplasm.
In conclusion, these results suggest that the C1b domain contains a structure that determines the Golgi localisation of PKCθ. Since also the PKCθRD fusion protein lacking the C2 domain (i.e. PKCθPSC1V3) is unable to induce apoptosis, this domain also plays a role in the apoptotic effect. C2 domains have previously been shown to mediate protein-protein

interactions [44], and based on that we speculate that the C2 domains are important for interactions with putative target proteins at the Golgi complex.
In paper 3 we aim to further pinpoint the structures within the PKCθC1b domain that mediate the Golgi localisation. To accomplish this, we compare the localisation of the C1 domains of several PKC isoforms in SK-N-BE(2)C neuroblastoma cells by confocal microscopy. Using a series of PKC-EGFP fusion proteins, we demonstrate that the tandem repeats of C1 domains from PKCα, PKCδ, PKCε, PKCη and PKCθ all localise to the Golgi. Thus, Golgi localisation is not isoform specific, and all isoforms are likely to have a Golgi targeting motif. The next set of experiments show that while the isolated C1a domains of the above isoforms localise throughout the cells, all isolated C1b domain to some extent are targeted to the TGN. This implies that there is a motif that determines Golgi localisation in the C1b domains, and that this motif is absent from the C1a domains.

To test this hypothesis, we align the sequences encoding the C1a and C1b domains of PKCα, δ. ε, η and θ and look for residues that are common among the Golgi localising C1b domains and absent in the C1a domain. We also study the published crystal structure of the PKCδC1b domain [14]. Based on these examinations, we find two candidate residues corresponding to Asp-245 and Met-266 in PKCδ. Both residues are located outside the hydrophobic part of the C1b domain, which conceivable is inserted in the membrane, and hence they are not involved in binding of TPA. Moreover, they have their side chains exposed on the protein surface, which is required for interactions with putative target molecules. In this context it is interesting that conservation of methionine on the protein surface has been proposed to imply a protein binding site [266].

We continue our work with PKCθ and generate expression vectors encoding the C1b domain, in which one or both of the corresponding amino acids, Met-267 and Glu-246, are mutated to glycine (referred to as PKCθC1bM267G, PKCθC1bE246G and PKCθC1bDM, respectively). Confocal analysis of SK-N-BE(2) cells expressing the fusion proteins shows that the wild type PKCθC1b and PKCθC1bE246G fusion proteins are both enriched in the TGN. In contrast, PKCθC1bM267G and the double mutant (DM) localise throughout the cells. All mutants respond to TPA-treatment and thus probably maintain their overall conformation.

Our previous results show that the C1b domain of PKCθ is required both for Golgi localisation and the apoptotic effects of PKCθRD. Hence, we next investigate if the abolished Golgi localisation observed by mutation of Met-267 is sufficient for inhibition of PKCθRD-induced apoptosis. We generate expression vectors encoding PKCθRD with mutations of Met-267, Glu-246 or both residues to glycine, fused to EGFP (denoted θRD-M267G, θRD-E246G and θRD-DM, respectively). Confocal images SK-N-BE(2)C expressing the fusion proteins confirm that a single mutation of Met-267 or concomitant mutation of both residues abrogates the Golgi localisation of PKCθRD, without affecting the ability of the proteins

to respond to TPA. Moreover, labelling of cells with fragmented DNA by TUNEL staining reveals that neuroblastoma cells expressing θRD-M267G and θRD-DM have a significantly lower rate of apoptosis than their Golgi-localising counterparts. We also confirm that this effect is not caused by higher expression of wild-type θRD-EGFP.

In conclusion, the results in paper 3 imply that Met-267 in PKCθC1b is important for Golgi localisation. This is further supported by the fact that all known Golgi-targeted PKC C1 domains contain a methionine at the corresponding site, which is absent from the non-Golgi localising C1 domains. Moreover, the C1 domain of RasGRP1, which also localises to the Golgi has a methionine in the corresponding site (Figure 6). The Golgi localising C1 domain of Munc-13 has a valine, while the C1 domain of β2-chimaerin and the C1a domain of PKD have leucines (other large hydrophobic amino acids), at the corresponding sites. Thus, an amino acid residue with these characteristics may be involved in interactions with a Golgi-specific structure.
The mutated and non-Golgi localising PKCθRD variants are unable to induce apoptosis, and hence there seems to be a direct correlation between Golgi localisation and apoptotic activity of PKCθRD. We hypothesise that Met-267 is required for association with a so far unidentified Golgi protein. We base this assumption on the fact that C1 domain containing proteins have been shown to directly interact with proteins [29, 30]. Since the methionine residue is located outside the hydrophobic part of the C1 domain, which is assumed to be inserted in the membrane, and the mutated fusion proteins still are able to respond to TPA, we do not believe that the residue is involved in general interactions with cellular membranes.

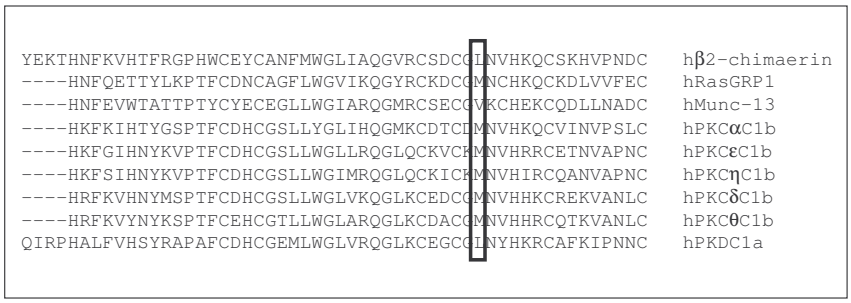

Figure 6 Alignment of sequences encoding the human C1 domains of β2-chimaerin (Swiss-Prot accession no P52757), RasGRP1 (NCBI accession no NP_005730), Munc-13 (NCBI accession no NP_006368), PKCαC1b, PKCεC1b, PKCηC1b, PKCδC1b, PKCθC1b and PKDC1a (NCBI accession no CAA53384). For the accession numbers of the PKC-C1b domains, see Schultz *et al* 2004.

The residue corresponding to Met-267 in PKCθ is required, but not sufficient, for Golgi localisation of C1 domains.

Since mutation of Met-267 in PKCθC1b is required for Golgi localisation, we hypothesise that mutation of the corresponding amino acid residue in PKCθC1a (i.e. Ala-195) can render this domain Golgi-localising. To test this, we generate an expression vector encoding the C1a domain of PKCθ fused to EGFP, in which Ala-195 is mutated to methionine (unpublished results). However, confocal analysis of SK-N-BE(2) cells expressing the fusion protein show that it resides throughout the cell, in a manner similar to the wild-type protein. Thus, although Met-267 is required for Golgi localisation of the C1b domains, mutation of the corresponding residue in PKCθC1a is not sufficient to make it Golgi localising.

Localisation of PKCε in neurite outgrowth (Paper II and III).

The localisation of PKC isoforms determines the enzyme's access to substrates and hence influences its biological activities. PKCε mediates neurite outgrowth in neuronal cell-lines and this ability is dependent on localisation of PKCε to the plasma membrane and/or the cortical cytoskeleton [216, 217].
During our investigations of the apoptotic effects of C_2-ceramide, we noticed that this treatment inhibits neurite outgrowth induced by PKCε. The RD of PKCε mediates the neurite-inducing capacity, which hence is independent of the kinase activity of PKCε [216]. Further experiments reveal that treatment with C_2-ceramide abolishes the neurite outgrowth in SK-N-BE(2) cells induced by expression of both PKCεFL and PKCεRD. Moreover, C_2-ceramide almost totally blocks the neurite-promoting effect of RA. The effect of RA can be inhibited by DN PKCε constructs and is thus likely dependent on PKCε [216]. In contrast, treatment with C_2-ceramide only slightly affects the neurites induced by treatment with the ROCK inhibitor Y-27632. Thus, C_2-ceramide is not a general inhibitor of neurite outgrowth but seems to have specific effects on PKCε-mediated processes.

Ceramide has previously been shown to induce subcellular translocation of PKC [28, 170, 171]. We therefore investigate the effects of C_2-ceramide on the localisation of PKCε as well as PKCδ and PKCθ. Although C_2-ceramide treatment does not affect the localisation of the latter isoforms, it clearly translocates PKCε from the cytosol to the perinuclear region in SK-N-BE(2) cells. Further examination of cells overexpressing PKCεRD shows that C_2-ceramide treatment induces a translocation of the fusion protein from the plasma membrane to the perinuclear region. Thus we conclude that C_2-ceramide might exert its neurite-suppressing effect by relocating PKCε and thereby inhibit its interaction with factors at the plasma membrane.

In paper 3 we further examine how Golgi localisation affects the neurite-inducing capacity of PKCε. As previously discussed, mutation of Met-267 is sufficient to abolish the Golgi localisation of PKCθC1b and we thus wanted to clarify whether a similar effect is observed

for PKCε. We mutate the corresponding residues in the C1b domain of PKCε (i.e. Asp-257 and Met-278) to glycine and examine the localisation of wild-type PKCε (PKCεWT) and the mutated fusion protein (PKCεDM) by confocal microscopy. In untreated cells, PKCεWT localises throughout the cytoplasm with a minor enrichment in the perinuclear region, while PKCεDM is distributed evenly in the cytoplasm. In response to TPA, both PKCε variants translocate to the plasma membrane. After treatment with C_2-ceramide, PKCεWT further translocates to the perinuclear region but, in contrast, PKCεDM still resides in the cytoplasm. Moreover, C_2-ceramide does not suppress the plasma membrane localisation of PKCεDM induced by TPA, which is observed for PKCεWT.

We next test whether PKCεDM also is more resistant to the neurite-inhibiting effect of C_2-ceramide. The experiment confirms that overexpression of PKCεWT induces neurites, an effect that is further potentiated by TPA. Treatment with C_2-ceramide inhibits the induction of neurites by PKCεWT, and a combined treatment with TPA cannot restore the neurite-inducing capacity. Overexpression of PKCεDM induces neurites just as potently as PKCεWT and treatment with TPA enhances this effect. However, compared to the PKCεWT-expressing cells, a significantly higher percentage of PKCεDM-expressing cells have neurites after C_2-ceramide treatment. Moreover, TPA-treatment partially restores the neurite inducing capacity of PKCεDM after addition of C_2-ceramide. Taken together, these results indicate that the Asp-257 and Met-278 residues are important for the ability of PKCεWT to respond to C_2-ceramide by relocating to the perinuclear region.

CONCLUSIONS:

- PKCδ and PKCθ act proapoptotic in neuroblastoma cells in response to treatment with TPA or C_2-ceramide.

- PKCα, PKCδ and PKCε are proapoptotic both in androgen-dependent and androgen-independent prostate cancer cells.

- The CD of PKCδ and the RD of PKCθ induce apoptosis in neuroblastoma cells in a caspase-3 dependent manner.

- PKC C1b domains localise to the Golgi complex, and the localisation depends on an amino acid residue with conserved properties, corresponding to Met-267 in PKCθC1b.

- The residue corresponding to Met-267 in PKCθC1b is required, but not sufficient, for Golgi localisation.

- The apoptotic activity of PKCθRD is directly correlated to its localisation to the Golgi complex.

- C_2-ceramide inhibits neurite outgrowth mediated by PKCε, conceivably by relocating the enzyme from the plasma membrane to the Golgi complex.

- The amino acid residues Asp-257 and Met-278 in PKCεC1b are important for the functional response of PKCε to C_2-ceramide.

Populärvetenskaplig Sammanfattning

Människokroppen består av triljoner celler som tidigt under fosterutvecklingen specialiseras (differentierar) för att bygga upp olika vävnader och organ. För att kroppen som helhet skall kunna fungera är det viktigt att cellerna kan kommunicera med varandra och anpassa sig till sin omgivning. Varje cell har därför ett komplext nätverk av signalvägar som känner av yttre faktorer, vidarebefordrar informationen inom cellkroppen och slutligen styr vilka proteiner som cellen ska tillverka. I tumörceller är signalvägarna emellertid satta ur spel vilket gör att cellerna kan dela sig ohämmat och överleva trots att omgivningen protesterar.

Enzymer är en typ av proteiner som fungerar som katalysatorer i kroppens kemiska processer. De är dessutom viktiga komponenter i cellens signalvägar, där de verkar genom att modifiera andra proteiner och därmed aktivera eller inhibera dem. I mitt forskningsarbete har jag studerat en familj av enzymer som kallas protein kinas C (PKC). PKC reglerar en mängd olika cellulära företeelser såsom differentiering, delning och cellulärt självmord. Det finns tio kända PKC medlemmar och de har namngivits efter grekiska alfabetet (α, βI, βII, δ, γ, ε, η, ι, ζ och θ).

PKC medlemmarna är strukturellt sett mycket lika och är uppbyggda av en katalytisk del samt en regulatorisk del. Den katalytiska delen binder och kemiskt förändrar andra proteiner, medan den regulatoriska delen styr aktiviteten hos den förra. Trots de strukturella likheterna kan de olika PKC varianterna styra helt skilda processer inom en cell, vilket man tror beror på att de skickas till olika platser i cellen och därmed modifierar olika proteiner. För att kunna generera mediciner som specifikt verkar på enskilda PKC medlemmar måste man därför förstå vad som styr var i cellen de hamnar (lokaliseras).

Syftet med min avhandling har varit att öka förståelsen för vad som bestämmer PKC varianternas lokalisering och vad det sin tur får för effekt på cellens beteende. Till största del har jag utfört mina experiment i celler som isolerats från barncancerformen neuroblastom. Neuroblastom uppstår i nervsystemet och dessa tumörceller har stannat i ett omoget stadium där de fortsätter att dela sig okontrollerat i stället för att specialiseras till nervceller eller begå självmord.

I delarbete I och II av min avhandling har jag därför undersökt om man kan förmå neuroblastomcellerna att begå självmord (apoptos) genom att öka deras tillverkning av enskilda PKC medlemmar. Rent praktiskt gör man detta genom att stoppa in fler genkopior av den aktuella PKC varianten i cellen och därmed tvinga den att producera mer av detta protein. Vi visar här att ökade nivåer av PKCδ och PKCθ får neuroblastomceller att vilja dö om man samtidigt tillsätter antingen ceramid eller forbolestrar (två typer av fetter som aktiverar PKC). En intressant aspekt av detta fynd är att självmordseffekten som erhölls av PKC varianterna inte berodde på deras förmåga att kemiskt förändra andra proteiner. När vi undersökte detta närmre upptäckte vi att den regulatoriska delen från PKCθ (PKCθRD) ensam kunde döda cellerna vilket inte tidigare upptäckts. I senare försök (delarbete I och III) såg vi att PKCθRD lokaliserades väldigt specifikt till en cell struktur som kallas Golgi apparaten. Golgi apparaten fungerar framförallt som cellens postkontor och det är här nytillverkade proteiner får en slags

adresslappar som gör att de senare hamnar på rätt ställe när de skickas vidare inom eller ut ur cellen. Genom en rad experiment kunde vi påvisa exakt vilka strukturer i PKCθRD som gjorde att proteinet i fråga hamnade i Golgi apparaten. Dessutom såg vi att PKCθRD måste lokaliseras till denna cellstruktur för att få cellerna att begå självmord. Tyvärr har vi inte lyckats visa varför PKCθRD får cellerna att begå självmord, men vi tror att det interagerar med andra proteiner i Golgi apparaten som sänder vidare dödssignalerna.

Tidigare resultat från vår forskargrupp har visat att PKCε varianten reglerar processer i neuroblastomceller som gör att de blir mer lika den specialiserade nervcellen. En karakteristisk egenskap hos mogna nervceller är att de har långa utskott (s.k. neuriter) som möjliggör kommunikation med andra nervceller. I experiment som ingår i delarbete II och III har vi visat att tillsatts av fettmolekylen ceramid hindrar PKCε från att generera neuriter på neuroblastomceller genom att flytta enzymet från cellens omgivande membran till Golgi apparaten.

I delarbete IV undersöker vi hur skillnader i nivåer av PKCα, δ och ε varianterna påverkar celldöd i två olika typer av prostatacancer celler. Normala celler i prostatan är beroende av könshormoner (androgener) för att kunna överleva. För att behandla prostatatumörer stryper man därför kroppens egen utsöndring av androgener, varpå många cancerceller dör. Tyvärr överlever ofta en andel tumörceller som är oberoende av androgener, varför det är viktigt att hitta nya vägar för att förhindra deras tillväxt. Tidigare studier har visat att PKCα och PKCδ gynnar celldöd i prostatacancer celler. När det gäller PKCε råder det emellertid delade meningar. En forskargrupp hävdar att PKCε ökar cancercellernas förmåga att överleva och dessutom bidrar till att göra tumörerna androgenoberoende, medan en annan grupp har visat att PKCε gynnar celldöd. När vi jämförde en androgenoberoende prostata celltyp med en androgenoberoende variant fann vi att båda uttryckte lika mycket PKCε samt att ökade mängder av PKCε gynnade celldöd. Dessa fynd talar mot att PKCε skulle öka tumörcellernas förmåga att överleva i avsaknad av androgener. I vår studie bekräftar vi även de tidigare resultaten att både PKCα och PKCδ får prostatacancerceller att begå självmord.

Sammanfattningsvis har jag genom mitt forskningsarbete identifierat specifika PKC varianter som förmedlar cellulärt självmord i neuroblastom och prostatacancer celler. Vidare har vi identifierat strukturer som medför att PKC varianter hamnar i Golgi apparaten, vilket gynnar celldöd utövad genom PKCθ, men missgynnar neuritutväxt via PKCε. Ökad kunskap om vilka PKC varianter som gynnar celldöd samt deras bakomliggande mekanismer kan i förlängningen leda till utveckling av mediciner som bromsar tumörcellernas tillväxt.

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