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PO Box 117
221 00 Lund
+46 46-222 00 00

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Roles of protein kinase C in cell death and breast cancer

Louise Cornmark



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DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at the main lecture hall, Jan Waldenströms gata 59, Skåne University
Hospital, Malmö. Friday 26th of April at 9:15 a.m.

Faculty opponent

Associate Professor Maria Shoshan, PhD
Department of Oncology-Pathology
Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden

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<p>Abstract</p> <p>Aberrant protein kinase C (PKC) activity and expression is implicated in different malignancies. To study the role of different PKC isoforms in breast cancer the expression of PKCα, δ and ϵ was evaluated in breast cancer tumors. In addition the effect of siRNA-mediated knockdown of the isoforms was studied in a global gene expression analysis. We found that high PKCα levels correlate with poor prognosis, high proliferation and estrogen receptor negativity. We have also seen that PKCα suppresses the expression of stanniocalcin-1 in breast cancer cells.</p> <p>Previous reports have shown that PKCδ is a survival factor in several breast cancer cells. Here we show that Smac, a proapoptotic protein, associates with PKCδ in many different cancer cell lines. Furthermore, the PKCδ-Smac association was dissociated upon paclitaxel treatment. Upon PKC activation with TPA the PKCδ-Smac complex was stabilized and the paclitaxel-mediated dissociation and death was suppressed. The decreased cell death could potentially be caused by a competition between PKCδ and XIAP for Smac binding. We also show that activation of PKC sensitizes some breast cancer cell lines to a Smac mimetic called LBW242, a small molecule that mimic the effect of Smac. We found that the TPA+LBW242-mediated cell death was dependent on TPA-induced TNFα production. In addition the combination of TPA+LBW242 enables complex II formation and caspase-3 cleavage, a probable cause of the concomitant cell death observed.</p>		
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Roles of protein kinase C in cell death and breast cancer

Louise Cornmark



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*Apoptosis, from Greek (ἀπόπτωσις) falling
off of petals from flowers or leaves from trees*

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Abbreviations

A1	Bcl-2 related protein A1	D	aspartic acid
Ala	Alanine	DAG	diacylglycerol
Apaf-1	Apoptotic protease activating factor-1	DD	death domain
aPKC	atypical PKC	DED	death effector domain
Apo3	apoptosis antigen-3	DIABLO	direct IAP binding protein with low pI
ATM	ataxia telangiectasia mutated	DMBA	7,12-dimethylbenz[α]anthracene
AVPI	Alanine valine proline isoleucine	DNA	deoxyribonucleic acid
Bak	Bcl-2 homologous killer	DR	death receptor
Bax	Bcl-2-associated X protein	EDAR	ectodysplasin A receptor
Bcl-2	B cell lymphoma 2	EMT	epithelial to mesenchymal transition
Bcl-W	Bcl-2 like 2 protein	ER	estrogen receptor
Bcl-XL	B-cell lymphoma extra-large	ERBB2	Avian erythroblastosis oncogene B 2
BH3	Bcl-2 Homology domain 3	FADD	Fas-associated death domain
Bid	BH3 interacting domain death agonist	Fas L	Fas ligand
Bik	Bcl-2 interaction killer	FEC	5-fluorouracil, epirubicin and cyclophosphamide
Bim	Bcl-2 interacting mediator of cell death	G	glycine
BIR	baculovirus inhibitor of apoptosis protein repeat	GSEA	gene set enrichment analysis
BLM	Bloom syndrome	HER2	human epidermal growth factor receptor 2
Bmf	Bcl-2 modifying factor	HM	hydrophobic motif
Bnip-3	Bcl-2/adenovirus E1B 19-kd protein-interacting protein 3	HOIL	haem-oxidized IRP2 ubiquitin ligase-1
BRCA	breast cancer gene	HOIP	HOIL-1L-interacting protein
C1-4	conserved region 1-4	Hrk	harakiri
CARD	caspase recruitment domain	IAP	inhibitor of apoptosis protein
CD95	cluster of differentiation 95	Ile	Isoleucine
cFLIP	cellular FLICE-like inhibitory protein	ILP2	IAP like protein 2
cIAP	cellular IAP	IP3	inositol trisphosphate
CMF	cyclophosphamide	IP3R	inositol triphosphate receptor
cPKC	classical PKC	K48	lysine 48
CRD	cystein rich domains		
CSC	cancer stem cell		

K63	lysine 63	Q	glutamine
LOH	loss of heterozygosity	Rb	retinoblastoma
LOX	lysylxygenase	RCC	renal cell carcinoma
M	methionine	RING	really interesting new gene
M1	methionine 1	RIP	receptor interacting protein kinase
MALT	mucosa-associated lympho ^{id} tissue	RNA	ribonucleic acid
Mcl-1	Myeloid cell leukemia sequence 1	Smac	second mitochondria-derived activator of caspases
ML-IAP	melanoma IAP	STC1	stanniocalcin-1
MOMP	mitochondria outer membrane permeabilization	TAB	TAK-binding protein
mTORC	mammalian target of rapamycin complex	TAC	docetaxel, doxorubicin and cyclophosphamide
MXI1	Max interactor-1	TAK	TGFβ-activated kinase
NAIP	neuronal apoptosis inhibitory protein	tBid	truncated Bid
NEMO	NFκB essential modulator	TGFβ	transforming growth factorβ
NFκB	nuclear factor kappa B	TIC	tumor-initiating cell
NGFR	nerve growth factor receptor	TM	turn motif
NHG	Nottingham histological grade	TMA	tissue microarray
NOXA	latin for damage	TNF	tumor necrosis factor
nPKC	novel PKC	TNFR	TNF receptor
PB1	phox bem1	TNFSF	TNF super family
PCR	polymerase chain reaction	TNFSFR	TNFSF receptor
PDK-1	phosphoinositide dependent kinase-1	TNM	tumor node metastasis
PI3K	phosphatidylinositol-3-kinase	TP53	tumor protein 53
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	TPA	12-O-tetradecanoylphorbol-13-acetate
PIP2	phosphatidylinositol 4,5-bisphosphate	TRADD	TNF receptor-associated death domain
PKC	protein kinase C	TRAIL	TNF-related apoptosis-inducing ligand
PR	progesteron receptor	TRAILR	TRAIL receptor
Pro	Proline	TWEAK	tumor necrosis factor-like weak inducer of apoptosis
PS	pseudo substrate	V1-5	variable region 1-5
PUMA	p53 upregulated modulator of apoptosis	Val	Valine
		VEGF	Vascular endothelial growth factor
		X	any amino acid
		XIAP	X-chromosome linked IAP

List of papers

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

Paper I

Lønne GK, **Cornmark L**, Zahirovic IO, Landberg G, Jirström K, Larsson C.
PKC α expression is a marker for breast cancer aggressiveness
Mol. Cancer. 2010 9:76

Paper II

Cornmark L, Lønne GK, Jögi A, Larsson C.
Protein kinase C α suppresses the expression of STC1 in MDA-MB-231 breast cancer cells.
Tumour Biol. 2011 Oct;32(5):1023-30

Paper III

Masoumi KC, **Cornmark L**, Lønne GK, Hellman U, Larsson C.
Identification of a novel protein kinase C δ -Smac complex that dissociates during paclitaxel-induced cell death.
FEBS Lett. 2012 Apr 24;586(8):1166-72.

Paper IV

Cornmark L, Holmgren C, Masoumi KC, Larsson C.
PKC activation sensitizes basal-like breast cancer cell lines to Smac mimetic-induced cell death
Manuscript

Breast cancer

Epidemiology and etiology

Breast cancer is the most common cancer among women world wide, with approximately 8000 newly diagnosed cases in Sweden each year and thus, represents almost 1/3 of all diagnosed cancers in women [1-3].

There are different factors that contribute to the overall risk of developing breast cancer. Some of the most common risk factors are early menarche, late menopause, nulliparity and late age at first childbirth [4,5]. Even though the geographical differences are decreasing there is still an increased risk for women in developed countries to develop breast cancer [5]. Risk factors with modest impact include the use of oral contraceptives or hormonal replacement therapy, whereas risk factors with a higher impact include a family history of breast cancer and previous benign breast disease [4-7].

Tumor progression

Timeline of breast tumor development

Breast tumor development is proposed to be a stepwise process with transformation of normal cells in the terminal ducts lobular units. Breast cancer can be initiated with the benign forms columnar cell hyperplasia and benign atypical hyperplasia. These do not necessarily continue beyond a benign form. However, there is an increased risk of developing into carcinoma *in situ* followed by invasive breast carcinoma [8].

Cancer stem cells and developmental origin of cancer cells

There are at least two different hypotheses for the development of breast cancer. These are the cancer stem cell (CSC, also called tumor-initiating cell, TIC) hypothesis and the developmental origin of cancer cell theory [9].

According to the CSC hypothesis a cancer cell arises from a transformed stem cell (also called progenitor cell) able to replicate and to differentiate into cells constituting the tumor bulk. Alternatively, cancer arises from a differentiated cell with acquired

stem cell characteristics. Thus the CSC would represent a minor subpopulation within a tumor more resistant to therapy and the driver of the tumor [9,10].

However, there is evidence that breast cancer arises from a differentiation program initiated by the mammary progenitor cell leading to the luminal progenitor and ending in the mature luminal cells. Breast cancer will then arise from any of the developing stages. Alternatively, more or less differentiated cells along the differentiated axis will acquire self-renewal abilities turning a differentiating cell into a cell with cancerous properties [9].

Hallmarks of cancer

The transformation of normal cells to cancer cells include genetic changes giving rise to a cell with one or more of the hallmarks of cancer described over a decade ago. These include 1) self sufficiency in growth signals, 2) insensitivity to anti-growth signals, 3) tissue invasion and metastasis, 4) limitless replicative potential, 5) sustained angiogenesis and 6) evading apoptosis [11]. Since 2000, new hallmarks as well as modifications of the old have been proposed. These include 7) deregulating cellular energy, 8) avoiding immune destruction, 9) tumor promoting inflammation and 10) genomic instability and mutations [12]. This thesis focuses on the hallmark cell death evasion in breast cancer.

Mutations

Mutations driving tumor transformation can occur in different ways. Aberrant regulation of proto-oncogenes due to gain of function mutations, amplifications or translocations drives cell growth, division and survival [13]. The mechanism by which mutations or deletions in tumor suppressor genes leads to loss of function commonly requires loss of heterozygosity (LOH). LOH requires that both alleles of a gene must be dysfunctional for the protein to be ineffective. This “two hit hypothesis” was first described for the retinoblastoma protein (Rb) in 1971 [14]. However, conflicting evidence to the “two hit hypothesis” was observed for the tumor suppressor genes *ATM* and *BLM* [15,16]. Today reports have shown that some tumor suppressor genes are haploinsufficient, meaning that only one hit is needed to initiate transformation of cells [17]. Modifying actions of gene expression without changing the DNA sequence known as epigenetic events can also influence tumor progression [18]. Hypermethylation of the promoter region of tumor suppressor genes have been reported to silence gene expression of eg the *BRCA1* gene [19]. Hypomethylation is believed to work in the opposite manner on proto-oncogenes and in addition contribute to chromosomal instability [20]. Other epigenetic events influencing tumorigenesis is the modulatory effects of micro RNAs [21].

The most common mutations or amplifications in breast cancer of proto-oncogenes are *PIK3CA* (encoding PI3K) and *ERBB2* (encoding HER2), which are reported in

36% and 20% of breast cancer cases, respectively [22,23]. Among commonly mutated or deleted tumor suppressor genes in breast cancer, *TP53* (encoding p53) and *RB* are observed in 37% and 20-35% of the cases, respectively [22,23]. In hereditary forms of breast cancer somatic mutations of the tumor suppressor genes *BRCA1* and *BRCA2* are the most common ones [24].

Classification of breast tumors

Prognostic and predictive markers

Prognostic markers predict patient outcome independent of treatment. Predictive markers intend to estimate patient response to a certain treatment. Biomarkers are thus predictive markers enabling personalized treatment. Some markers have both prognostic and predictive value [25].

Histological classification

Breast cancer is a heterogeneous disease that, based on morphological and histological features, can be divided in two major groups, invasive breast carcinoma and non-invasive carcinoma *in situ*. Both the invasive and the non-invasive tumors can be of ductal or lobular origin. Breast cancer of ductal origin is the most common among both invasive and non-invasive breast carcinoma. Among invasive breast cancers, the ones of ductal origin comprises approximately 75% and the lobular only 5-15% of the cases [26,27]. In addition, several other subgroups of invasive breast cancer exist but constitute a small proportion of the invasive breast cancers. With a few exceptions, histological subtypes have low prognostic and/or predictive value [26].

Histological grade

Nottingham histological grade (NHG) is, similar to tumor type, based on histological and morphological features but differs from tumor type by conferring more prognostic value. It assesses the degree of differentiation and is based on three features including tubular count, nuclear pleomorphism and mitotic counts. These features are rated 1-3 enabling a classification system with grades I-III according to differentiation where a tumor of grade I is well differentiated, grade II is moderately differentiated and grade III is poorly differentiated [28].

Tumor stage

Progression of the breast cancer can be measured by taking different parameters into account using a TNM stage. TNM is based on tumor (T) size, lymph node (N)

involvement and if distant metastasis (M) is present or not. TNM stage serves as a guideline of what treatment regiments to employ. The stages range from I-IV taking all parameters into account [29,30].

Immunohistochemical markers

To help determine adjuvant therapy, estrogen receptor (ER) and progesterone receptor (PR) status is evaluated with immunohistochemistry. If positive, endocrine treatment such as tamoxifen can be employed. Immunohistochemical staining of HER2, and in some cases *in situ* hybridization of the HER2 gene transcribed by *ERBB2*, is analyzed to determine whether targeted treatment with trastuzumab (Herceptin[®]) should be initiated. These three proteins are routinely evaluated in the clinic and are predictive markers indicative of treatment outcome [27].

Molecular subclassification

Results based on analyzes of global gene expression have enabled the categorization of breast cancer into different molecular subgroups [31]. Throughout the past 13 years adjustments to these subgroups have been made. Today luminal A, luminal B, HER2-enriched, basal-like, claudin-low and sometimes the normal breast-like group are recognized as distinct molecular signatures, referred to as the intrinsic subtypes of breast cancer [9]. These subgroups have revealed differences in survival and response to treatment and thus, contain both prognostic and predictive information [32,33].

The majority of the tumors of luminal A and B subtype are ER and PR positive and HER2 negative. Luminal A tumors are, compared to luminal B, of lower histological grade and associated with good prognosis whereas the luminal B subgroup is of high grade and have worse prognosis [32,33]. The HER2-enriched tumors can be either ER positive or negative, are of high histological grade and despite the HER2 targeting drug trasuzumab (Herceptin[®]) patients in this group usually have worse prognosis [9,33]. The majority of the basal-like tumors are ER, PR and HER2 negative. Many of the claudin-low tumors are also triple negative and in addition have increased levels of EMT markers and a stem cell-like phenotype [32]. A triple negative phenotype is often linked to a worse prognosis which could be due to the fact that these tumors are of high histological grade and unresponsive to endocrine- and/or HER2 targeted therapy [9,32].

Treatment

Surgery and adjuvant treatment

Treatment of primary breast cancer includes surgery, radiotherapy, chemotherapy and targeted therapy. What treatment regiments to use depends on different factors mentioned earlier. However, the first line of treatment is surgery and no adequate

substituting treatment exists. Surgery involves mastectomy (whole breast removal) or breast conserving surgery [27,34]. Chemotherapy is seldom warranted after mastectomy of tumors smaller than 5 cm and negative lymph node involvement but routinely follows breast conserving surgery and mastectomy in cases where the tumor is larger than 5 cm. Targeted therapy such as endocrine treatment can be initiated in ER and PR positive tumors with eg estrogen receptor inhibitors such as tamoxifen or aromatase inhibitors. If HER2 amplification is established, targeted therapy with the monoclonal antibody trastuzumab (Herceptin[®]) can be administered. In triple negative tumors, surgery and radiotherapy is followed by chemotherapy often consisting of a combination of drugs such as FEC (5-fluorouracil, epirubicin and cyclophosphamide), TAC (docetaxel, doxorubicin and cyclophosphamide) or CMF (cyclophosphamide, methotrexate, 5-fluorouracil) [27,35].

Clinical trials

Before a new potential anti-cancer drug can be used in the clinic it needs to be thoroughly investigated in a stepwise manner. This starts with pre-clinical testing consisting of basic research with animal studies. If successful, clinical trials involving patients can be initiated in phases I-III before being approved for use. There are many different clinical trials currently ongoing. One type of compound tested in clinical trials, of special interest for this thesis, are the apoptosis facilitators Smac mimetics which will be discussed later [36].

Stanniocalcin

First discovered in fish stanniocalcin (STC) was found to regulate Ca^{2+} levels as well as inorganic phosphorus resorption [37,38]. The human ortholog was found by two separate groups in the mid-1990s [39,40]. A few years later a second STC family member was discovered [41-43]. The first one discovered got the name STC1 and the second one discovered was named STC2.

STC1 is a protein of 247 amino acids with 11 cysteine residues located N-terminally that are important in creating a homodimer [44]. Other sites important for posttranslational modifications are the two N-terminally glycosylation consensus sequences, NDS (Asn-Asp-Ser) and NST (Asn-Ser-Thr) [44]. This allows STC1 to be secreted from cells as a glycosylated homodimeric protein [45].

STC1 is expressed in a wide variety of cells. It is reported in neurons [46], adipocytes [47], striated muscle [48] and megakaryocytes [49]. STC1 is also found in bone [49], kidney and ovaries [50] as well as in mammary tissue [51,52].

The function of STC1 is not completely known. STC1 has been shown to stimulate the electron transport chain [53], to uncouple the mitochondria [54] and to activate mitochondrial anti-oxidant pathways [55,56].

Elevated STC1 expression has been detected in human samples from different malignancies such as colorectal cancers, hepatocellular carcinoma, non-small cell lung cancer, ovarian cancer and leukemia [57]. Reports of STC1 and its function in breast cancer are scarce but the expression of STC1 seems to be downregulated in BRCA1-negative cells [51] and the expression is higher in ER-positive than in ER-negative tumors [52]. STC1 levels also seem to be up-regulated in late metastasis [58]. We show in paper II that STC1 expression can be repressed by PKC α in breast cancer cells.

Protein kinase C

Protein kinase C (PKC) is a family of highly related serine and threonine kinases that convey a vast number of signals upon activation [59-61]. PKC was first discovered by Nishizuka and co-workers in the late 1970s [62,63] and soon thereafter it was found to be activated by tumor promoting phorbol esters [64-66]. This discovery led to many investigations of PKC and its possible role, and thus also as a possible target, in oncogenesis.

Structure

Based on structure and mode of activation, the PKC family members can be divided into three different groups. These are the classical PKCs (cPKC) comprising α , β I, β II and γ ; the novel PKCs (nPKC) containing δ , ϵ , η , θ and the atypical PKCs (aPKC) consisting of ζ , ι/λ [59] see figure 1. All PKCs contain four conserved (C) regions C1-C4 and five variable (V) regions V1-V5 and together they constitute an N-terminal regulatory and a C-terminal catalytic domain [59]. The regulatory domain of the cPKCs and the nPKCs contain two typical C1 domains that can bind and are activated by diacylglycerol (DAG) [67,68] or by phorbol esters, a functional analogue of DAG often used in PKC activation [64-66,69]. In addition to DAG, the cPKCs also require Ca^{2+} binding to the C2 domain for activation [59,68]. nPKCs contain a C2-like domain unable to bind Ca^{2+} and does not require it for activation. The aPKCs contain an atypical C1 domain that does not bind DAG, has no C2 domain but instead a phox bem-1 (PB1) domain and can be activated by other proteins containing a PB1 domain such as PAR6-CDC42 complex, MEK and p62 [70]. Due to their differences in structure and mode of activation aPKCs will not be the focus in this thesis.

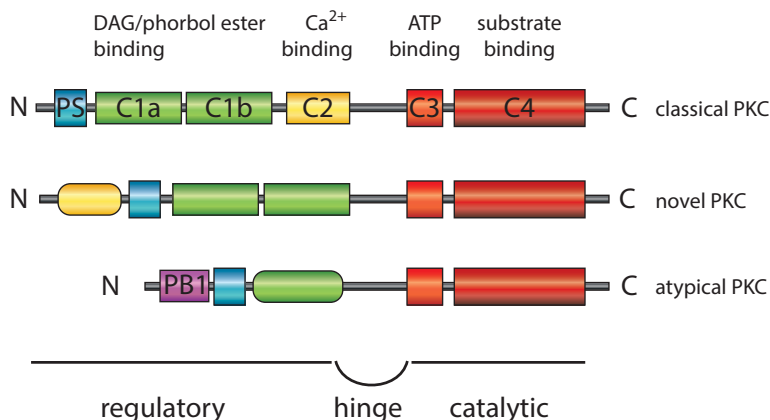


Figure 1. Structure of PKC

The family of PKCs can be divided into three different classes: the classical, the novel and the atypical PKCs. Common for all PKC family members is that they contain a regulatory N-terminal and a catalytic C-terminal domain connected by a hinge region.

Regulatory domain

The regulatory domain contains regions controlling the activity of PKCs. It is the domain with the most variation between PKC family members [61,71]. The pseudo substrate (PS) autoinhibits the protein by binding to the substrate recognition motif [59,72]. The C1 and C2 domains are responsible for binding different types of activators such as diacylglycerol and/or phorbol esters for C1 and Ca^{2+} for C2 [61] see figure 1. These domains will be further discussed below.

PS

The PS resides at the N-terminal part of the regulatory domain of PKCs. The PS contains a sequence that resembles a PKC substrate but with an alanine in place of the serine or threonine phosphoacceptor site of a substrate [71-73]. The PS keeps the enzyme in a locked conformation inhibiting catalytic activity [71].

C1

The C1 domain is defined as a cysteine rich region that in the classical and the novel PKCs is present in tandem repeats and referred to as C1a and C1b. The C1 domain can be further divided into typical and atypical, where the typical bind DAG or phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA), whereas the atypical do not [74]. The cysteine residues are important for the C1 structure. Three cysteines together with one histidine form two Zn^{2+} -ion coordination sites making up the structure of a globular protein with a binding pocket for DAG or phorbol esters [69,75,76]. Upon DAG or phorbol ligand binding, the top third of the C1 domain

requires a new lipophilic surface important for membrane association enabling insertion of the C1 domain into membranes [69, Newton, 1997 #87,77]. Hurley and coworkers proposed that a protein surface change with stabilized membrane-insertion is of importance for PKC activation [69,77]. This was further strengthened by the fact that the C1 domain of aPKCs do not bind DAG, possibly since atypical C1 domains lack important amino acids in the DAG-binding site or hydrophobic residues important for membrane insertion [69]. Thus, it is not the lack of two tandem C1 domains that makes the aPKCs incapable of binding DAG or phorbol esters. Furthermore, the non-kinase proteins such as chimaerins, Munc13s, RasGRPs with one or more typical C1 domain(s) have the capacity to bind DAG [69].

C2

A functional calcium-binding C2 domain is present only in cPKCs and has the capacity to bind 2-3 Ca^{2+} -ions [76]. Increased intracellular Ca^{2+} levels results in binding of Ca^{2+} to negatively charged aspartic residues in the C2 domain when the protein is still in the cytosol. Upon Ca^{2+} -binding, cPKC obtains an increased affinity to negatively charged phospholipids in the membrane. At the membrane Ca^{2+} serves as the bridge between C2 and phosphatidylserine [78]. The calcium binding region 3 can bind a third Ca^{2+} -ion stabilizing the C2 domain-membrane complex. The PKC protein can thus reside longer at the membrane allowing the C1 domains to encounter DAG and complete the activation of the enzyme [69,76]. This is the basis of the C2-C1 theory [69,79]. The nPKCs have a C2-like domain without Ca^{2+} binding capacity [61].

Hinge region

The hinge region, also called the V3 region, is located between the regulatory and the catalytic domain in PKC [71]. The V3 region of PKC δ , PKC θ , PKC ϵ and PKC ζ , contain a caspase-3 recognition site DXXD, where X is any amino acid and D is Aspartic acid, and are targets for caspase-dependent cleavage [80]. Upon cleavage the catalytic fragment is freed from the autoinhibitory regulatory domain. Cleavage at the hinge region activates PKCs with a catalytically competent catalytic domain. PKC δ , not requiring activation loop phosphorylation for its activation, and PKC ϵ which retains activation loop phosphorylation prior to cleavage both have competent catalytic fragments [71].

Catalytic domain

The catalytic domain contains the ATP-, substrate binding- and catalytic sites as well as sites important for maturation of the enzyme, the activation loop, the turn motif (TM), and the hydrophobic motif (HM).

ATP binding site

The greatest sequence homology among the PKC isoforms resides within the catalytic domain with approximately 65% sequence homology between PKC β , ϵ and θ compared to a less than 50% overall homology between the same isoforms [61, Steinberg, 2008 #13]. The ATP-binding loop contains a glycine-rich consensus sequence of GXGXXG, which is a structural hallmark of protein kinases [71]. One Lysine, 17 amino acids downstream of the ATP binding site of all PKCs, is important for kinase activity. Mutation of this lysine demonstrates its importance, and is often experimentally utilized, since it creates a kinase inactive mutant [71].

Substrate binding site

PKC recognizes substrates for phosphorylation in its C4 domain where the substrate binding site is located [81]. The substrate of PKC is serine or threonine typically in the context of a basic sequence. PKC has a maximal catalytic rate of approximately 10 reactions per second [81]. A more unique preferred substrate sequence surrounding the serine and threonine site has been proposed for different PKC isoforms [82]. However, there are features common for optimal substrates for all PKCs [82].

Regulation of PKC

Post-translational modifications of the PKC protein are part of a maturation process required for PKC to become competent and capable of responding to second messengers such as DAG. The phosphorylation of three conserved sites, the activation loop located in the C4 domain, the TM and the HM both located in the variable region 5 (V5) are responsible for achieving maturation [60,83] see figure 2.

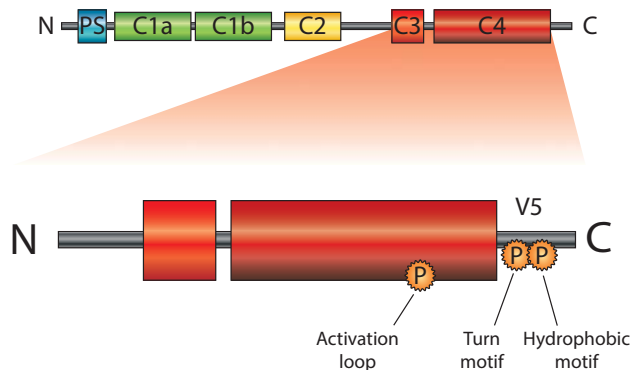


Figure 2. Phosphorylation sites important for PKC maturation

The phosphorylation sites of the activation loop, the turn motif and the hydrophobic motif in the C4 domain and V5 region, important for PKC maturation, are depicted.

Maturation and phosphorylation

Activation loop

In all cPKCs and nPKCs, except PKC δ , phosphorylation of the activation loop is required for a mature and functional PKC. PKC is proposed to translocate to the plasma membrane shortly after translation where the phosphoinositide dependent kinase-1 (PDK-1) phosphorylates the activation loop of cPKCs [84-86]. This is made possible by the open conformation PKC adopts after translation exposing the activation loop for phosphorylation by PDK-1 [83]. Phosphorylation of the activation loop precedes and is necessary for the phosphorylation of the next conserved regions, the TM and HM [87].

Turn- and hydrophobic motif

The mammalian target of rapamycin complex 2 (mTORC2) have been reported to control the phosphorylation of the TM. Depleting cells of mTORC2 results in low levels of PKC indicating the importance of phosphorylation at the TM for PKC stability [88]. Both the mTORC2 and the mTORC1 have been implicated in phosphorylation of the HM in PKCs [89] which is facilitated by binding to the heat shock protein HSP90 [90]. The phosphorylation of this site seems to be important for keeping the kinase in a closed inactive state protected from dephosphorylation [60,91,92]. The phosphorylation of the TM and HM are important for PKC stability and intact phosphorylation status of the enzyme as well as for kinase activity and substrate phosphorylation [88].

Activation

The mode of activation differs somewhat for cPKCs and nPKCs. The C2 domain of nPKCs (see previous section on C2) are insensitive to Ca²⁺ and does not require it for activation as the cPKCs do, however nPKCs have stronger affinity for DAG possibly evolved as a compensatory mechanism to its Ca²⁺-insensitive C2 domain [93].

One mode of cPKC activation can be exemplified by ligand stimulation of the Gq protein coupled receptor. This leads to phospholipase C β (PLC β) activation and subsequent hydrolysis of PIP2 into DAG and IP3 [94]. IP3 binds to IP3 receptors of the endoplasmic reticulum releasing Ca²⁺ into the cytosol [94]. Two Ca²⁺-ions bind the C2 domain of the mature and competent cPKC residing in the cytosol. The Ca²⁺-bound C2 domain retains an altered electrostatic potential attracting it towards membranes with enhanced affinity for anionic phospholipids such as PIP2 and phosphatidyl serine where a third Ca²⁺-ion binds and stabilizes the C2-membrane association [76,95]. This prolongs the duration of the enzyme at the membrane enabling DAG binding to the C1 domain [76]. Upon DAG binding the C1 domain

obtains a lipophilic surface enabling further insertion into the lipid layer of the membrane. The membrane committed enzyme retains enough energy to expel the PS from the substrate binding cavity thus allowing substrate phosphorylation [81]. The nPKC that do not have a Ca^{2+} -binding C2 domain have a stronger affinity for DAG which is enough for expulsion of the PS from the substrate binding cavity [76,78,96,97]. Once activated, the translocation of PKC from one cellular compartment to another has been suggested by the Mochly-Rosen group to be mediated by different isoform specific receptors for activated C kinases (RACK)s [98,99]. Thus, RACKs are thought to position an active PKC in close proximity to its substrate [100].

Degradation

The PKCs have relatively long half-lives, up to days for cPKCs. However, treating cells with phorbol esters yielding a sustained PKC activation results in the rapid degradation of PKC, eventually leading to the depletion of the enzyme from the cell [83].

Depending on activating stimuli, PKCs can be degraded in different ways. One pathway wherein the activated membrane bound PKC is ubiquitinated and targeted for proteasomal degradation is reported for TPA activated PKCs. A second pathway of degradation involves a pool of caveolae-associated active PKCs that are internalized, dephosphorylated and finally degraded by proteasome-independent mechanisms [83,101,102].

PKC in sickness and in death

PKC in breast cancer

Mutations of PKCs are not common and point mutations of PKC α have been described only in certain cases of pituitary and thyroid cancers [103]. However, to date no report on PKC mutations have been made for breast cancer. On the other hand changes in expression levels or activation status of different PKC isoforms have been reported in different cancers including breast cancer [94].

The mammary epithelium undergoes many changes from development to involution during which fluctuating levels of PKCs have been reported. High levels have been observed at pregnancy for PKC α , δ , ζ , η , lower levels of PKC α , δ , ϵ but continuous expression of PKC η at lactation and high levels of PKC δ , ζ , η during involution [104,105].

PKC α

The role of PKC α in breast cancer is complex since reports of it acting as both a tumor suppressor and tumor promoter exists. PKC α is reported to be downregulated when comparing breast cancer with normal breast tissue [106] and decreasing with tumor grade [107] whereas others report an upregulation in breast cancer tissue with HER2 amplifications [108]. *In vitro* studies of stably PKC α -overexpressing MCF-7 cells resulted in an increased proliferation and anchorage-independent growth, decreased ER expression, loss of epithelial morphology and increased vimentin expression. All of which are changes indicative of a more aggressive phenotype [109]. Other studies of MCF-7 cells overexpressing PKC α reported an association with multidrug resistance [110] as well as induction of tamoxifen resistance [111].

We reported of a negative correlation between PKC α and ER expression in paper I. In addition, only a few breast cancers were PKC α positive [112]. Our results can thus help explain the somewhat contradicting reports of low PKC α expression levels in breast tumors [106,107] with the *in vitro* reports linking PKC α overexpression to a more aggressive phenotype. Further, in a murine mammary model a specific PKC α inhibitor almost completely reduced metastasis which correlated to increased survival of the animals. The inhibitor was reported to decrease intravasation by decreased

matrix metalloproteinase-9 activity, cell migration and a decrease in NF κ B activity [113].

PKC δ

Most reports of PKC δ involve its role as an anti-tumorigenic isoform owing to the fact that it is involved in caspase-3 cleavage (see next section).

However, there are also reports of PKC δ having pro-tumorigenic effects. Overexpression in mouse mammary cells increased anchorage-independent growth [114]. Furthermore, studies indicate that PKC δ can promote proliferation [115] and metastasis development [116-118]. PKC δ has also been shown to contribute to anti-estrogen resistance in mammary tumors [119]. Additional observations further indicate that removal of PKC δ is sufficient to initiate cell death in certain breast cancer cell lines [120]. A recent study reported that PKC δ correlated with poor prognosis in HER2-positive human breast cancer. In addition, PKC δ was reported to be required for HER2-driven proliferation [121].

PKC ϵ

PKC ϵ is often described as the oncogenic PKC isoform. It has been proposed to be a marker of breast cancer aggressiveness. High levels of PKC ϵ have been found to correlate with HER2 expression, tumor grade and ER negativity as well as poor patient survival [122]. When overexpressed in a mouse mammary tumor-derived cell line, PKC ϵ -enhanced survival against apoptotic insults was reported as well as increased colony formation. This implicates PKC ϵ in the development of murine tumor progression [123]. Although most studies indicate a proliferating anti-apoptotic effect of PKC ϵ the enzyme is also described to have antiproliferative activity when treating cells with tamoxifen [124].

Other PKC isoforms

PKC η seems to be regulated by estradiol in the ER positive breast cancer cell lines MCF-7 and T47D. Moreover, induced expression of PKC η in MCF-7 cells decreased the apoptotic markers caspase-7, caspase-9 and PARP-1 cleavage [125]. Furthermore, expression levels of PKC η correlates with multi drug resistance receptors [126]. When it comes to PKC θ it has been described as having the ability to derepress cRel and initiate NF κ B signaling by repressing ER α synthesis [127]. PKC β is often considered a growth promoting isoform. Its inhibition reduced growth of MCF-7, MDA-MB-231 and BT-474 breast cancer cells whereas its overexpression led to increased cyclin D1 levels, a protein involved in cell cycle progression [128].

PKC δ and cell death

PKC δ is often described as the pro-apoptotic member of the PKC family. During apoptosis, PKC δ can be activated by proteolysis. This was reported to be due to a caspase-3 recognition motif in the hinge region of PKC δ consisting of the amino acids DMQD [80,129]. The cleavage of PKC δ generated a free catalytic active fragment with ability to induce nuclear fragmentation and cell death [129]. Overexpression of the catalytic PKC δ fragment induces cell death and translocates the PKC δ fragment to the nucleus or mitochondria in different cell lines [130,131] This is in accordance with different apoptotic stimuli resulting in a translocation of endogenous PKC δ to these compartments [132,133].

Many different cell death inducing stimuli have been reported to induce caspase-3 dependent cleavage of PKC δ , such as etoposide [133,134], γ -irradiation [80], UV-radiation [135] and mitomycin [136] to name a few. It has also been shown that inhibiting PKC δ decreases the cell death induced by different agents [134,135]. Furthermore, the PKC δ knockout mouse was reported to be defective in caspase-3 activation upon etoposide treatment [137]. Thus, this indicates that PKC δ can have an effect both upstream and downstream of caspase-3.

Cell death

The first scientifically documented descriptions of cell death were reported in the 19th century by Carl Vogt. Although no mention of cell death was made, observations of “destroyed” or “disappeared” cells were reported [138,139]. With improved laboratory techniques spontaneous loss of cells as an important event to balance mitosis was observed in the 1960s [139,140]. Because of its important kinetic significance it was proposed in 1972 that the process of cell death be given a name of its own. “Apoptosis”, a word derived from Greek describing the falling off of petals from flowers or leaves from trees was chosen [140]. With further gained insights into the molecular pathways that regulate and execute cell death since 1972, many different molecular definitions of different cell death subroutines have been postulated [141]. However, to limit the scope of this investigation apoptosis will be the focus of this section.

Apoptosis

Before advanced biotechnological approaches of determining cell death were adapted, apoptosis was described as morphological changes. By analyzing different cells exposed to varying forms of insult in an electron microscope Kerr, Wyllie and Currie characterized apoptosis with the following features: cell and nuclear shrinkage, chromatin condensation, separation from neighboring cells, membrane blebbing and formation of apoptotic bodies terminated by phagocytosis and degradation by other cells [140]. These features still hold true today. However, since 1972 biochemical methods for assessing cell death have been developed which help assist in delineating specific subroutines of cell death and also enables quantitative measurements of apoptosis [141].

There are two main pathways mediating apoptosis. The intrinsic (also known as mitochondrial) and the extrinsic (also known as receptor mediated) pathway which both converge in a programmed manner at caspase activation with subsequent cell death [142,143].

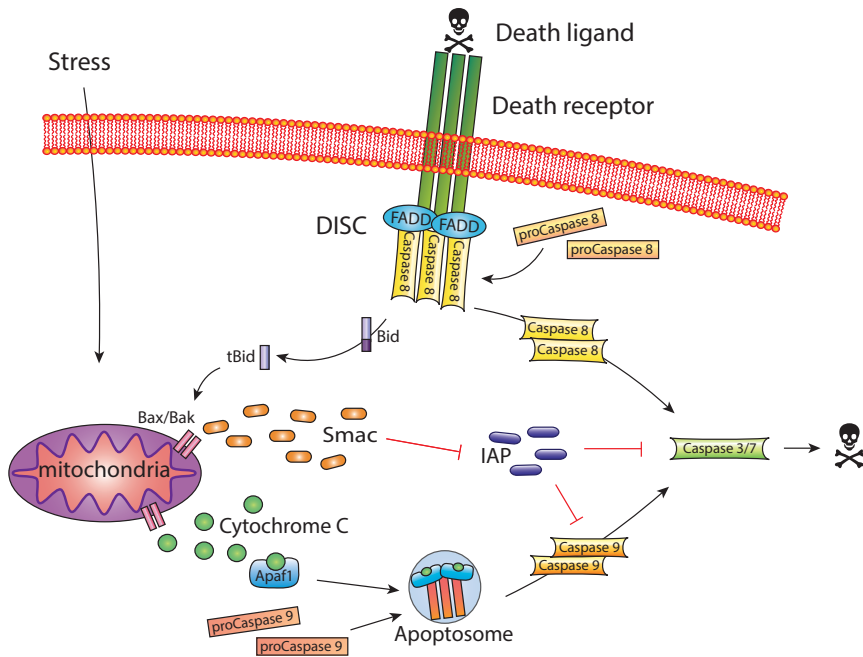


Figure 3. The extrinsic and intrinsic apoptotic pathway

The intrinsic apoptotic pathway is initiated by a stimuli leading to MOMP and the release of pro-apoptotic proteins, some of which forms the apoptosome with caspase-9. Other pro-apoptotic proteins such as Smac blocks IAPs, relieving inhibition of caspases and facilitating cell death. The extrinsic apoptotic pathway is initiated by ligand binding of its death receptor resulting in receptor oligomerization. Adaptor proteins and caspase-8 or -10 are recruited to the receptor creating the DISC. Both the apoptosome and the DISC are platforms for initiator caspase activation, followed by activation of executioner caspases-3 and -7 resulting in apoptosis.

Intrinsic pathway

The major effector of the intrinsic apoptotic pathway is the mitochondria and is thus also sometimes referred to as the mitochondrial pathway. Irradiation, chemotherapeutic toxins or growth factor deprivation are examples of factors that can initiate mitochondrial outer membrane permeabilization (MOMP) through members of the Bcl-2 family [142,144]. MOMP leads to the release of cytochrome c and second mitochondria-derived activator of caspases (Smac) also known as direct inhibitor of apoptosis protein with low pI (DIABLO) to the cytosol [144,145]. Cytochrome c will together with the adaptor protein apoptotic protease-activating factor 1 (Apaf-1) recruit the initiator pro-caspase-9 forming the apoptosome. Pro-caspase-9 is then autoproteolytically cleaved to its active form which can continue activating executioner caspases-3 and -7 [146,147] ultimately resulting in proteolytic cleavage of protein substrates and cell death [141,142,148,149] see figure 3.

Regulation of MOMP by the Bcl-2 family members

Regulators of MOMP are members of the B cell lymphoma-2 (Bcl-2) family. They are either anti- or pro-apoptotic. The pro-apoptotic proteins can be subdivided into effectors consisting of Bax and Bak and BH3-only proteins Bad, Bid, Bim, Bmf, Bnip3, Hrk, NOXA and PUMA. The molecular events resulting in Bax/Bak-mediated MOMP are just now beginning to unfold. Two different models have been proposed. The indirect activator and the direct activator-derepressor model [150].

In the indirect activator model the anti-apoptotic Bcl-2 family members: Bcl-2, Bcl-X_L, Bcl-W, Mcl-1 and A1 bind the constitutively active pro-apoptotic Bcl-2 family members Bax and Bak. BH3-only Bcl-2 family members compete with the binding to the anti-apoptotic family members releasing active Bax and Bak. The released and active Bax and Bak are free to homodimerize creating pores resulting in MOMP [150,151].

In the direct activation-derepressor model some BH3-only proteins, called direct activators, activate Bax and Bak. Bid and Bim have been reported to function as direct activators [152] and anti-apoptotic Bcl-2 family members inhibits the function of these proteins by sequestering them thereby inhibiting activation of Bak or Bax [153]. In this model, a second subset of BH3-only family members function as sensitizers by neutralizing the anti apoptotic Bcl-2 family members [150]. Some have the ability to sensitize all anti-apoptotic Bcl-2 family members such as PUMA, Bim and Bid, whereas the others have higher specificity for inhibiting certain anti-apoptotic Bcl-2 family members [150,154].

Extrinsic pathway

The death receptors (DR)s of the tumor necrosis factor receptor super family (TNFRSF) mediate the extrinsic apoptotic pathway which is therefore also referred to as the death receptor mediated pathway. Upon ligand binding, the receptors are aggregated and assemble proteins in a death-inducing signaling complex (DISC). The DR Fas (also known as CD95) recruits Fas-associated death domain (FADD) that in turn recruits the pro-caspases-8 and/or -10. Caspase-8 and -10 are initiator caspases of the extrinsic pathway and have autoproteolytic capacity when in close proximity, resulting in caspase cleavage and activation. The active initiator caspases will subsequently activate executioner caspases-3 and/or -7 completing the extrinsic apoptotic cell death pathway [142,149] see figure 3.

Type I and type II cells

The two apoptotic pathways are not strictly separated from each other. Crosstalk between the extrinsic and intrinsic pathway is made possible upon extrinsic caspase-8 activation. One substrate of caspase-8 is the pro-apoptotic Bcl-2 family member Bid. When Bid is cleaved by caspase-8 to an active truncated form (tBid), tBid is capable of initiating MOMP [150] se figure 3.

Cells that undergo apoptosis upon an extrinsic stimulus without requiring involvement of the intrinsic pathway, exemplified by Fas ligand treated lymphocytes and thymocytes, are called type I cells [155]. However, some cells rely upon intrinsic pathway activation and MOMP after an extrinsic death stimulus in order to initiate apoptosis. Hepatocytes and pancreatic β -cells are examples of these kind of cells referred to as type II cells [155,156]. Recent research indicate that inhibiting or removing XIAP in these cells removes the Bid dependence of the apoptotic inducing capacity of Fas ligand [157].

Death receptors

The DR receptors are part of the TNFRSF with 29 structurally similar receptors. Common for all is a significant homology in the extracellular domain with cysteine rich domains (CRD) important for ligand specificity. Among the TNFRSF there are subsets of 8 receptors with a cytoplasmic death domain (DD). These receptors are thus called death receptors and constitute TNFR1, Fas (CD95/DR2), TWEAK (DR3/Apo3), TRAIL1 (DR4), TRAIL2 (DR5), DR6, EDAR and NGFR [158]. The ones most studied in cell death and as possible targets in cancer treatment are TNFR1, Fas, TRAIL1 and TRAIL2. TNFR1 will be further discussed below. For more information about the other DRs, a review by Mahmood & Shukla is recommended [158].

TNFR1

There are two mammalian TNF receptors, the TNFR1 and the TNFR2. TNFR2 lacks a DD, is not classified as a DR and will therefore not be discussed further. TNFR1 contains a DD and is by definition a DR. However, TNFR1 activation triggers NF κ B signaling, often associated with inflammation, cell survival and proliferation but can also play an important role for mediating cell death [159,160].

The fate by which the TNFR1 signals is determined by the proteins available and recruited to the receptor upon ligand binding.

Complex I

Upon binding of the ligand tumor necrosis factor α (TNF α) to the TNFR1, the death domain of the receptor rapidly recruits other proteins with death domains such as TNFR1-associated via death domain (TRADD) and receptor interacting protein 1 (RIP1) [161,162]. TRADD recruits TNFR-associated factor 2 (TRAF2), cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 [162]. These proteins make up complex I [163] see figure 4.

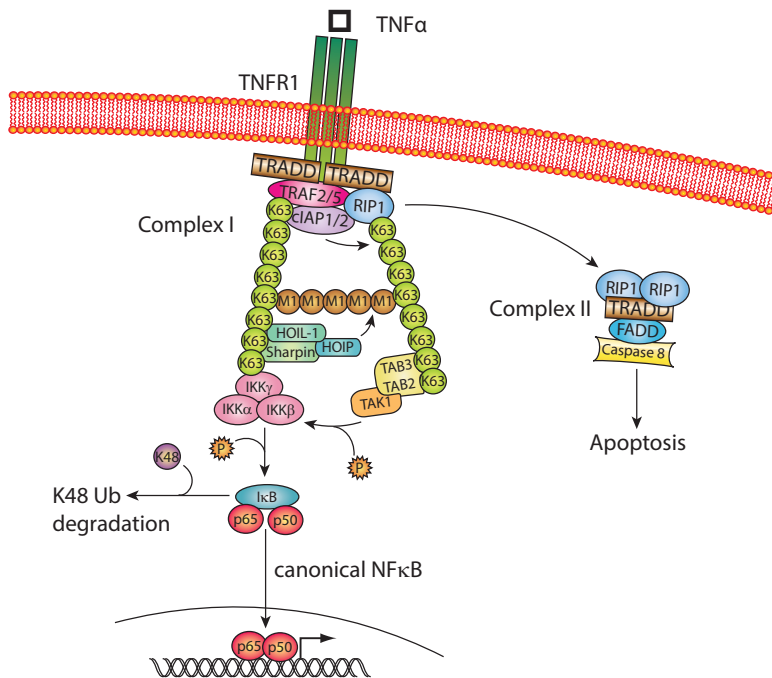


Figure 4. TNFR1 signaling

Upon TNF α ligand binding to the TNFR1, complex I is formed in the presence of cIAP1 and cIAP2 mediating canonical NF κ B signaling. However, in the absence of cIAP1 and cIAP2 complex I cannot form. In stead, RIP1 and TRADD recruit FADD and caspase-8 forming complex II. If caspase-8 is activated, apoptosis can be initiated.

Once complex I is formed the E3 ubiquitin protein ligases cIAP1 and cIAP2 add lysine 63 (K63) ubiquitin linear chains to RIP1. TRAF2, also an E3 ubiquitin ligase, does not function as the E3 ligase for RIP1 [162]. The ubiquitinated proteins form a scaffold to which TGF β -activated kinase 1 (TAK1), TAK1 binding protein 2 (TAB)2, TAB3 and members of the inhibitor of NF κ B kinase (IKK) complex IKK α , IKK β and IKK γ (also known as NEMO) are recruited. The complex is further

stabilized with linear methionine 1 (M1)-linked ubiquitin by the LUBAC complex consisting of HOIP, HOIL-1 and Sharpin [164-166]. TAK1 together with TAB2 and TAB3 activates IKK β of the IKK complex. Once active, IKK β phosphorylates and targets the inhibitory NF κ B subunit (I κ B) for lysine 48 (K48) mediated ubiquitin degradation releasing the p50 and/or RelA (also known as p65) NF κ B dimers. Once liberated the NF κ B subunits dimerize, translocate to the nucleus and initiate transcription of pro-inflammatory and anti-apoptotic genes [142] see figure 4.

Complex II

In the absence of cIAP1 and cIAP2, RIP1 is not ubiquitinated and the scaffold required for successful NF κ B signaling is lost. Instead the DD-containing proteins RIP1 and TRADD dimerize with the DD of FADD. FADD contains both a DD and a death effector domain (DED) enabling recruitment and homologous interaction with the DED on caspase-8 [167]. The closely related kinase RIP3 can also be recruited to RIP1. Together all these components form the complex II. By inactivating the RIP kinase activity and autoproteolytic cleavage, caspase-8 can initiate apoptotic signaling, see figure 4.

Modulators of cell death

There are many different components involved in an apoptotic response. Proteins initiating cell death must be activated and proteins tightly regulating activation of cell death must be inhibited or bypassed. This balance of pro- and anti-apoptotic proteins is necessary for the survival of a cell, in order not to spontaneously die or keep on living forever.

Caspases

One family of proteins with a central role in apoptosis is the caspase family. Caspases are cystine proteases that cleave after an aspartate residue in their substrates [168]. In humans 11 different caspases have been identified of which some are involved in other processes than apoptosis, such as inflammation [169,170]. The caspases involved in apoptosis can be divided into two different groups. The initiator caspases, includes caspase-2, -8, -9 and -10 and the executioner caspases includes caspase-3, -7 and -6. Caspases are produced as inactive zymogens and must be activated by cleavage in order to function as a protease. The activation of an executioner caspase is carried out by an initiator caspase. The initiator caspases have auto-proteolytic activity but often require the assembly of different proteins in order to initiate the auto-proteolytic activity [170].

IAP

The family of inhibitor of apoptosis protein (IAP) is involved in regulating whether a cell will live or die by a stress stimuli or insult. The first human IAP was described in 1995 [171], today there are eight known human IAPs; neuronal apoptosis inhibitory protein (NAIP also known as BIRC1), cellular IAP1 (cIAP1 also known as BIRC2), cellular IAP2 (cIAP2 also known as BIRC3), X chromosome-linked IAP (XIAP also known as BIRC4), survivin (also known as BIRC5), ubiquitin-conjugating BIR domain enzyme apollon (apollon also known as BRUCE or BIRC6), melanoma IAP

(ML-IAP also known as livin or BIRC7), IAP like protein 2 (ILP2 also known as BIRC8). The characteristic feature of an IAP is one or three baculovirus IAP repeat (BIR) domains [149,162]. Other domains that some of the IAPs share are the RING domains that in XIAP, cIAP1, cIAP2 and ML-IAP possesses E3 ubiquitin ligase activity [149,162,172] and a CARD domain that is unique for cIAP1 and cIAP2 and is suggested to mediate protein-protein interactions [173].

Functions of IAPs

Apoptotic signaling pathways converge at caspase activation, making the regulation of these proteases important for cell survival. IAPs regulate both initiator and executioner caspases either directly or indirectly [149,162]. Below, XIAP, cIAP1 and cIAP2 will be discussed in detail.

XIAP

Studies show that although some of the other IAP family members may bind to caspases, XIAP is the only IAP with inhibitory capacity [174-176]. XIAP binds to caspase-3 with the linker region N-terminally of BIR2 but both the linker region and BIR2 are needed for caspase-7 inhibition [176-178] whereas caspase-9 is inhibited with the BIR3 domain [176,179,180]. The BIR1 domain does not seem to be involved in caspase inhibition [162].

cIAPs

In 2007 cIAPs were revealed to play an essential role in apoptotic signaling separated from direct caspase inhibition. Vucic's and Silke's group reported that cIAP1 and cIAP2 were important regulators in directing the TNFR1 signal to complex I (see previous section on TNFR) by activating the canonical NF κ B pathway. It was reported that in the absence of cIAP1 and cIAP2 complex I could not form and that proteins present at the receptor were free to form complex II, with FADD, RIP1 and caspase-8, competent to induce apoptosis [181-183].

By functioning as the E3 ubiquitin-protein ligase of NF κ B-inducing kinase (NIK) cIAP1 and cIAP2 are further responsible for the constant degradation of NIK. When cIAP1 and cIAP2 are absent NIK is accumulated, activated and non-canonical NF κ B signaling is initiated. One of the target genes of non-canonical signaling is TNF α . Thus, TNF α forms a positive feedback loop enhancing the stimulation of the TNFR1. The signaling mediated through the TNFR1 in the absence of cIAPs is changed, like flipping a switch, from the pro-survival signaling complex I to the death signaling complex II.

Smac/DIABLO

In 2000, two research groups, independently of each other, discovered a protein with the capacity to inhibit IAPs and facilitate apoptosis. Xiadong Wang's group named the protein second mitochondria-derived activator of caspases (Smac) [184], while David Vaux's group named it direct IAP binding protein with low pI (DIABLO) [185]. The two groups were working with the same protein. Thus, today the name of the gene is *DIABLO* and the protein is usually referred to as Smac or Smac/DIABLO.

Smac encodes 239 amino acids which produces a 27 kD big immature protein. The first 55 N-terminally amino acids contain a mitochondrial localization sequence (MLS). The MLS directs pro-Smac to the mitochondria where, after the 55 amino acids are cleaved off, the mature and functionally active 25 kD form of Smac is released into the cytosol upon MOMP [184-186] see figure 5.

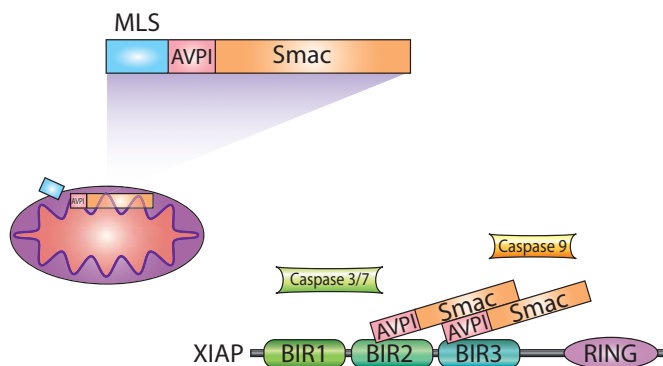


Figure 5. Smac structure and function

Newly synthesized Smac contains a mitochondria localization sequence (MLS). The MLS sequesters Smac to the mitochondria where the MLS is cleaved off creating mature Smac with an N-terminal amino acid sequence of AVPI. Once released from the mitochondria the AVIP is responsible for binding to the BIR2 and BIR3 domain of XIAP blocking inhibition of caspases.

The effect of Smac

Once released to the cytosol, mature Smac has a new N-terminal end containing a four amino acid long sequence proven to be essential for its function. The AVPI (Ala, Val, Pro, Ile) sequence is responsible for the binding to and inhibition of members of the IAP family [179,184,185]. Smac has been reported to bind to XIAP, cIAP1, cIAP2, survivin, ML-IAP and Apollon [184,185,187-190].

Smac homodimerizes through a hydrophobic interface forming a stable protein dimer that is essential for its activity [191,192]. Both the BIR2 and BIR3 domain of XIAP

needs to be inhibited by Smac in order to relieve caspase-7 and -9 of XIAP-mediated inhibition for [193].

Smac has the ability to initiate auto-ubiquitination leading to degradation of cIAP1 and cIAP2 whereas other IAPs such as XIAP and ML-IAP are suggested to be blocked through direct binding counteracting their functions [194]. However, ML-IAP and other IAPs have been proposed to inhibit Smac by degradation [189,195,196].

Other IAP binding proteins

Apart from Smac, other IAP binding proteins such as high temperature requirement protein A2 (HtrA2) [197], apoptosis related protein in the TGF β signaling pathway (ARTS) [198] and XIAP-associated factor 1 (XAF1) [199] have been identified.

Smac and IAPs in cancer

The aberrant expression and/or function of IAPs and Smac have been reported for many different human cancers. Genetic investigations suggest that cIAP1 and cIAP2 are oncogenes. Since 2000, many publications have reported that the expression levels of IAP proteins and their antagonists correlates with clinical parameters and cancer prognosis in different retrospective trials. Here only a few studies will be mentioned. For a more extended investigation an excellent review on the topic by Simone Fulda & Domagoj Vucic is recommended [149].

Gene amplification, translocations and deletions

The 11q21-23 amplification harboring both cIAPs is seen in various types of tumors including cervical cancer, esophageal carcinoma, glioblastoma, medulloblastoma, hepatocellular carcinoma, liver cancer, non-small-cell lung cancer (NSCLC), small cell lung cancer and pancreatic cancer implicating cIAPs in tumor progression. The t(11;18)(q21;q21) translocation where the BIR domains of cIAP2 is fused with the paracaspase mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT) resulting in the cIAP2-MALT protein and a constitutively active NF κ B signaling pathway is frequently seen in MALT lymphoma [200,201]. Deletion at chromosome 12q24.31, the locus for Smac/DIABLO, has been observed in Mycosis fungoides a subtype of cutaneous T cell lymphoma [202].

Leukemia

There are different reports on IAPs as a prognostic marker in different types of leukemia. In two AML studies low levels of XIAP expression correlated with better overall survival and response to treatment [203,204]. Overexpression of cIAP2 in AML was identified to predict poor overall survival in a three-gene expression

signature. In addition, high levels of Smac expression was reported to be an independent prognostic factor associated with longer overall survival and higher complete remission rate [205].

Renal cell carcinoma

An increased ratio of XIAP:Smac expression have been implicated in the progression of renal cell carcinoma (RCC) [206]. Furthermore, XIAP expression was reported to be increasing with advancing tumor stage, tumor dedifferentiation and aggressive tumor growth [206,207]. In addition XIAP has been reported to be an independent prognostic parameter in RCC [207].

Breast cancer

In breast carcinoma, nuclear but not cytoplasmic expression of XIAP correlated with shorter overall survival [208]. A strong correlation between the intensity of XIAP immunostaining and the grade of invasive ductal carcinoma or ductal carcinoma *in situ* was reported in another study. In addition, this study reported of less XIAP staining on average in lobular compared with ductal carcinoma [209]. Furthermore, a study of invasive ductal carcinoma revealed prognostic value of XIAP expression. High XIAP protein expression correlated to low overall survival and reduced disease-free survival [210]. Smac levels were shown to negatively correlate with tumor stage and invasive growth [211].

Re-establishing cell death

Evading cell death

Since evasion of cell death is a hallmark of cancer and many of the resistant and refractory cancer cases have defects in their cell death pathways one attractive target for cancer therapy is to re-establish a functional cell death program in order to defeat cancer [212].

There are many different ways by which a cancer cell can evade a death stimulus. In different hematological malignancies there are reports of mutations in the CD95 gene [213,214]. Dysfunctional transport of TRAILR1 and 2 to the plasma membrane as well as decreased expression of the TRAILRs that confers resistance to TRAIL have been reported in different types of cancer [215,216]. Overexpression of endogenous inhibitors of caspase-8 such as cFLIP inhibits signal transduction through the death receptor pathway and is reported in different malignancies [217,218]. Caspase-8 inactivating mutations are very rare. However, alternative splicing resulting in a caspase-8 lacking the kinase domain and epigenetic silencing of the gene is seen in different cancer types such as neuroblastoma [219,220].

The intrinsic pathway is often dysfunctional in many types of cancer. In different types of leukemia an increased ratio of Bcl-2:Bax is correlated to tumor cell survival and apoptosis resistance [221,222]. The anti-apoptotic Mcl-1 is reported to be important in regulating disease progression and outcome in chronic lymphocytic leukemia [223].

As discussed in previous chapter IAPs and their endogenous inhibitors seem to play a role in various types of cancer. It is thus clear that different areas of cell death can be targeted for therapy development and the following passages will mention this area of research

Smac mimetics

When the human IAP antagonist Smac was discovered [184,185] the functionally important region comprising the four N-terminally amino acids AVPI was subsequently revealed [191,224,225]. This evoked an interest in developing small

molecule antagonists of IAPs by mimicking the AVPI sequence. Early studies of Smac based peptides showed that they could effectively block IAP-caspase interactions on a cellular level and sensitize glioma in xenografted mice to pro-apoptotic stimuli [187,226]. These studies used Smac-based peptides which do not possess the pharmacological properties necessary for therapeutic agents. Thus, the development of small molecule Smac mimetics was initiated [227]. One of the developed small molecule Smac mimetic compound is the LBW242, also used in the investigations of paper IV. LBW242 is a monovalent Smac mimetic that has been used in different investigations of Smac mimetic function. In different in vivo studies it has been shown to potentiate death activating stimuli [228,229]. In a recent investigation of Smac mimetic function, Smac mimetics were proposed to induce a conformational change of cIAP1. The conformational change enabled RING dimerization and E3 ligase activity of cIAP1 implying a degradative function of Smac mimetics on IAPs [230]. This is in line with publications showing degraded cIAPs upon Smac mimetic treatment [231-233]. In addition to monovalent, there are also bivalent Smac mimetic compounds consisting of two monovalent Smac mimetic molecules connected through a chemical linker [227,232].

The first clinical trial of a small molecular Smac mimetic compound started in 2008 and since then 10 different trials have been initiated. Only two of these were initiated prior to 2010 and are now in phase I or phase II clinical trials [36].

Present investigation

Aims

1. In paper I the aim was to elucidate the role of PKC in breast cancer by analyzing its relation to ER-, PR-status, proliferation, histological grade, occurrence of node or distant metastasis and prognosis
2. In paper II the aim was to analyze the genetic changes upon different PKC isoform depletion in MDA-MB-231 cells, and with the help of pre-existing gene sets elucidate whether the depletion of a specific PKC isoform is correlated with a specific signature
3. In paper III the aim was to investigate possible association partners to the PKC δ isoform and to study the implications of these interactions
4. In paper IV the aim was to study the impact of the Smac mimetic compound LBW242 together with the PKC activator TPA on different cancer cells

Paper I

PKC α expression is a marker for breast cancer aggressiveness

Aberrant protein kinase C signaling can result in different pathological states. However, whether different PKC isoforms conferr predictive or prognostic value has not been evaluated. Thus, in paper I our aim was to elucidate the role of PKC α , PKC δ and PKC ϵ in breast cancer.

Tumor material

To reach this aim the presence of the PKC isoforms α , δ and ϵ were investigated in tissue micro arrays (TMA)s from two different primary breast cancer cohorts and evaluated along with established clinicopathological parameters. Since cross reaction when analyzing different PKC isoforms is a notorious problem, several batches of antibodies were tested and evaluated for specificity of the individual isoform by using siRNA and overexpression of each isoform (Figure 1A-B).

The cells positive for antibody staining from cohort II (Figure 1C-J) indicated that the PKC isoforms investigated were present in the cytoplasm. The majority of the breast cancer specimens were not positive for PKC α staining. This is supported by previous findings of PKC α being downregulated in breast cancer compared to normal tissue [106,107] (Table 4). In the TMA for both cohort I and cohort II PKC α was negatively correlated with ER, PR and positively correlated with Ki67 and tumor grade (Table 3). This is in agreement with a report of increased PKC α levels in HER2 amplified tumors [108], tumors that often are ER and PR negative [9]. No correlation was seen for the other clinicopathological parameters investigated (nodal status and distant metastasis). PKC δ and PKC ϵ did not correlate with any of the parameters investigated neither in cohort I nor in cohort II (in cohort II only PKC α and ϵ were evaluated). When classifying the tumors in cohort II based on histological type, high PKC α histological staining intensity was overrepresented among medullary carcinomas (Table 4).

When analyzing at the patient material, patients with PKC α -negative tumors were associated with significantly improved 10-year breast cancer-specific survival compared to patients with PKC α -positive tumors. After uni- and multivariate analysis adjusting for age at diagnosis, tumor size, NHG, node status and ER expression the association between PKC α positivity and a poor 10-year breast cancer-specific survival was independent of established prognostic parameters.

Breast cancer cell lines

We evaluated PKC expression in different breast cancer cell lines and found that PKC α expression was high in MDA-MB-231 cells compared to MCF-7 and MDA-MB-468 cells. PKC α could not be detected in T47D cells. MCF-7 cells showed the highest expression of PKC δ whereas the PKC ϵ expression levels seemed to be similar in all analyzed cell lines (Figure 3A). MDA-MB-231 expressed the highest levels of PKC α and also showed the highest proliferation rate (Ki-67 staining) and growth rate, whereas T47D lacking PKC α had significantly lower levels of Ki-67 staining, in line with the tumor data.

Next, the influence of PKC activity on cell proliferation was studied. Our results indicate that TPA stimulation cannot induce cell proliferation under sub-optimal conditions (such as growth in serum free medium). The proliferation of the cancer cells under normal conditions could not be inhibited by using a classical PKC inhibitor Gö6976 (Figure 4A-C). This indicates that PKC α activity does not seem to be critical for growth under normal conditions.

The more specific inhibition of PKC α with siRNA targeted knockdown decreased cells in S-phase, especially cells grown under sub-optimal conditions (Figure 5C). This indicates that PKC α may be redundant for cell proliferation during normal

conditions but under sub-optimal conditions PKC α may play an important role in driving the cell through proliferation.

The involvement of PKC α in tumor migration and metastasis was further investigated by testing the migratory capacity of breast cancer cells after inhibiting PKC or knocking down PKC α with siRNA. Both a pan-inhibitor as well as a cPKC inhibitor significantly decreased the migratory capacity as demonstrated by decreased wound closure in a scratch wound healing assay. Only when mediating a complete silencing of PKC α , as in MCF-7 cells, a concomitant decreased migratory capacity of the cells was seen (Figure 6A-E). Thus, for breast cancer cell migration PKC α activity seems to be important. Corroborating results were reported by the Mochly-Rosen group where PKC α -specific inhibition decreases tumor dissemination in a mouse mammary cancer model [113].

Many studies on PKC involve different inhibitors. However, the results from these studies can be difficult to interpret since many of the inhibitors only are designed to inhibit a certain group of PKCs whereas the supposedly specific ones may have off target effects. Si-targeted downregulation of specific PKC isoforms is therefore an alternative. Previous findings revealed that siRNA targeted knock down of PKC δ and ϵ increased cell death of MDA-MB-231 and MDA-MB-468 cells [120]. This together with the results of PKC α in paper I led us to further investigate the isoform specific roles of PKC α , δ and ϵ in breast cancer by siRNA mediated knockdown followed by global gene expression analysis with microarray. The results, especially for siPKC α , were further examined in paper II.

Paper II

Protein kinase α suppresses the expression of STC1 in MDA-MB-231 breast cancer cells

In paper II the mechanisms mediating the phenotypical effects of PKC isoforms in breast cancer cells were further investigated. PKC α , PKC δ or PKC ϵ were downregulated in MDA-MB-231 cells. Global gene expression was thereafter analyzed. The genes were ranked from the genes being the most upregulated to the genes that were the most downregulated upon siPKC α treatment compared to control. The ranked list was compared to pre-existing gene data sets in a gene set enrichment analysis (GSEA). Only gene sets with a false discovery rate of <0.01 were investigated (Figure 1). Among the gene sets investigated three hypoxia-induced gene

sets were enriched among genes whose expression was increased following PKC α downregulation.

Thus, PKC α -regulated genes were negatively correlated with hypoxia-driven genes. The removal of PKC α could possibly result in hypoxic features.

Therefore, mRNA expression of four hypoxia driven genes from the gene lists *VEGF*, *STC1*, *MXI1* and *LOX* [234-236] were analyzed to elucidate whether they were PKC α -regulated genes. However upon PKC α downregulation, only STC1 could be readily downregulated with different oligos targeting PKC α (Figure 3 A-C). We continued to investigate how PKC α and hypoxia may influence STC1. There was neither an additive nor a synergistic effect of the combination of downregulated PKC α and hypoxic treatment (Figure 4A).

STC1 is expressed in a wide variety of tissues. STC1 has been shown to stimulate the electron chain transport [53], to uncouple the mitochondria [54] and to activate mitochondrial anti-oxidant pathways [55,56]. However, reports of STC1 function in breast cancer are scarce but its expression seems to be downregulated in BRCA1-negative cells [51] and the expression is higher in ER positive than in ER negative tumors [52]. STC-1 levels also seem to be up-regulated in late metastasis [58]. To gain further insight in a potential role for STC1 in breast cancer, the protein expression was investigated in different breast cancer cell lines (Figure 5A). The fact that STC1 is excreted as a homodimeric glycosylated protein could contribute to the many different bands visible when blotting for STC1. The cell line with high PKC α levels MDA-MB-231 had lower levels of a STC1 species above the 40 kD marker. On the other hand the smaller species was abundant in MDA-MB-231. The expression of the smaller approximately 32 kD fragment (the size of STC1) increased during both PKC α downregulation as well as during hypoxia (figure 5B-C).

Our study implicates PKC α in the suppression of STC1 mRNA expression and indicates an altered transcription profile by PKC α in breast cancer cells. As reported in paper I high levels of PKC α are correlated to poor patient outcome. Other reports of STC1 and breast cancer show increased STC1 levels with BRCA1 overexpression [51] as well as a positive correlation of STC1 and ER status in breast carcinomas [52]. Thus, for breast cancer, low STC1 could implicate an aggressive phenotype.

Paper III

Identification of a novel protein kinase C δ -Smac complex that dissociates during paclitaxel induced cell death

In Paper III we studied the PKC isoform δ more closely. PKC δ is often described as the pro-apoptotic PKC isoform. However, in a previous study downregulation of PKC δ led to cell death in different breast cancer cell lines [120]. Therefore we decided to investigate putative binding partners of PKC δ that could reveal some of the functions of PKC δ . A screen for PKC δ -interacting proteins by mass spectrometry analysis of trypsin-digested proteins present in PKC δ precipitates was performed. Smac, one of the proteins of putative relevance was identified. To further confirm the interaction, both immunoprecipitation (IP) of Smac and reciprocal IP of PKC δ was performed and further established the interaction (Figure 1A-D). The interaction was not confined to breast cancer cell lines but was seen in many other cancer cell lines although in some cell lines the interaction was weak (Figure 1F). Further, we found the interaction to be specific for the PKC δ isoform (Figure 1E).

The importance of AVPI

To determine the Smac-PKC δ interaction site on Smac, different mutants of Smac was transfected into MCF-7 cells and IP of endogenous PKC δ was made. The IP revealed that the same binding sequence that has been described as the amino acid sequence necessary for IAP inhibition, the AVPI (Ala-Val-Pro-Ile) [224,225,237] is needed for a functional Smac-PKC δ association (Figure 1G).

Since Smac is released from the mitochondria upon different apoptotic stimuli the PKC δ -Smac interaction upon paclitaxel treatment was investigated. In paclitaxel sensitive breast cancer cell lines the interaction was disrupted upon paclitaxel treatment (Figure 2A-E) concomitant with the release or cleavage of other apoptotic markers.

The role of the kinase activity for the PKC δ -Smac complex was investigated by using either the PKC activator TPA or the PKC inhibitor GF109203X. Upon PKC activation with TPA, the PKC δ -Smac complex was stabilized and the paclitaxel-mediated disassociation was decreased. In addition this stabilization coincided with increased viability and decreased cell death, an effect that was abolished when adding a PKC inhibitor (Figure 3A-C).

One explanation as to how a maintained PKC δ -Smac association decreases cell death could possibly be by a blocking effect of PKC δ on the AVPI sequence of Smac,

inhibiting released Smac from binding to IAPs. An effect that was indeed seen when overexpressing PKC δ RD together with Smac and XIAP (Figure 4C-D).

Paper IV

PKC activation sensitizes basal-like breast cancer cell lines to Smac mimetic-induced cell death

In Paper IV we continue to look into cell death pathways by utilizing a Smac mimetic. As the name implies Smac mimetics are small molecules mimicking the four amino acids AVPI of endogenous Smac. Smac mimetics are thought to facilitate cell death in two ways, by blocking IAPs inhibitory effect on caspases [184,185] and by removing cIAP1 and 2 to redirect TNFR1 signaling from NF κ B to pro-apoptotic signaling [181,182]. Since we have previously (see paper III) seen an association between PCK δ and endogenous Smac we wanted to further investigate how a Smac mimetic compound, LBW242, would influence TPA treated cancer cells. One reason for using TPA is that it has been reported to induce TNF α production [238,239] a prerequisite in Smac mimetic sensitive cells to initiate cell death [181,182].

The combined effect of TPA and LBW242 was investigated in 13 different cancer cell lines. Of the 13 cancer cell lines investigated three breast cancer cell lines, one of which is sensitive to Smac mimetic as a single agent, decreased in viability (Figure 1A) and increased in cell death (Figure 1D-E) when the combination of TPA and LBW242 was administered. The decreased viability was dependent on PKC activity since a rescue in viability was established when using the pan-PKC inhibitor GF109203X. Common for the three cell lines is that they are all of the basal like phenotype.

Complex II formation

Next we wanted to investigate the mechanisms mediating TPA+LBW242 induced cell death. Smac mimetic sensitive cells have been reported to initiate complex II formation (containing RIP1 FADD and caspase-8) where caspase-8 is activated leading to subsequent apoptosis [181,182]. Therefore we investigated the effect of a caspase inhibitor in the presence of TPA+LBW242. The pan-caspase inhibitor zVADfmk was able to restore viability of the cells (Figure 2A). Further, when caspase-8 was immunoprecipitated other proteins of the complex II were co-immunoprecipitated upon TPA+LBW242 treatment in MDA-MB-468 cells (Figure 2B-C). This indicates that complex II formation is initiated upon TPA+LBW242 treatment. Next, we wanted to examine caspase activation as a possible mediator of

TPA+LBW242 induced cell death. Immunoblotting revealed cleaved caspase-3 upon TPA+LBW242 treatment (Figure 2D).

TNF α production

Smac mimetic sensitive cells are dependent on TNF α production and blocking TNF α with antibodies can rescue Smac mimetic sensitive cells from cell death [181,182]. In addition TPA has been reported to mediate NF κ B activity leading to TNF α production [238-240]. Thus we next investigated the involvement of TNF α .

When removing TNF α from the medium with TNF α targeting antibodies there was a decreased cell death upon TPA+LBW242 treatment (Figure 3A-B). When further studying both mRNA and protein levels after TPA+LBW242 treatment an increase in TNF α levels was seen by TPA treatment alone in the Smac mimetic insensitive cell lines. This indicates that the TPA+LBW242-mediated cell death is dependent on TPA-induced TNF α production

To further investigate the isoform responsible for the TNF α production downregulation of the novel PKC δ , PKC ϵ and the classical PKC α were made. Interestingly, the removal of any of the isoforms drastically decreased TPA-evoked TNF α production. However, only in cells with downregulated PKC δ TPA was unable to increase TNF α levels.

Conclusions

In this thesis we have gained knowledge of the PKC isoforms PKC α and PKC δ . We have also seen that PKC activity can influence the function of a Smac mimetic in breast cancer cells.

We conclude that:

- PKC α is a marker for breast cancer aggressiveness (paper I)
- PKC α suppresses the expression of stanniocalcin-1 (paper II)
- PKC δ forms a complex with Smac that dissociates upon paclitaxel treatment (paper III)
- Upon PKC activation the PKC δ -Smac complex is stabilized and the paclitaxel mediated dissociation and death is suppressed (paper III)
- PKC activation sensitizes certain breast cancer cell lines to the Smac mimetic LBW242-induced cell death (paper IV)
- TPA+LBW242-mediated cell death is dependent on TPA-induced TNF α production (paper IV)
- The combination of TPA and LBW242 initiates complex II formation and caspase activation (paper IV)

Populärvetenskaplig sammanfattning

För att cellerna i kroppen ska fungera krävs det att proteinerna i cellerna betar sig som de ska, dvs att de signalerar på ett korrekt sätt. Det finns en mängd olika signalvägar i en cell och lika många olika mekanismer som styr signalvägarna. Mycket av det som sker i cellen regleras på ett eller annat sätt utifrån cellens genetiska kod som finns i DNA.

DNA:t innehåller all cellens information och om DNA:t blir skadat, i form av mutationer, kan det bidra till att cellen börjar producera defekta proteiner eller inga protein alls. Konsekvensen av ett defekt protein kan innebära felaktig signalering i cellen som bidrar till att den utvecklar canceregenskaper. Sådana canceregenskaper är exempelvis okontrollerad delning och/eller förmågan att undvika att dö. Cancer är en mångfacetterad sjukdom och det finns många olika signalvägar och proteiner som kan vara defekta i olika typer av cancer.

I den här avhandlingen har rollen för signalproteinets proteinkinas C (PKC) studerats i bröstcancer. PKC är en familj med flera familjemedlemmar med olika egenskaper men med den gemensamma förmågan att fosforilera andra protein och påverka deras egenskaper.

PKC α , aggressiva bröstcancertumörer och stanniocalcin-1

I paper I använde vi oss av tumörmaterial från olika bröstcancerpatienter för att studera hur uttrycket av PKC såg ut i brösttumörer. Vi såg att en PKC familjemedlem, PKC α , var uttryckt i tumörer med mer aggressiva egenskaper. Vidare såg vi i paper II att samma familjemedlem påverkade uttrycket av vissa gener däribland stanniocalcin-1 (STC1). Den exakta funktionen för STC1 är ännu okänd men STC1 tros kunna påverka olika förlopp i bröstceller som både kan påverka överlevnad men också död.

Man brukar tala om att det finns sex olika områden som är karakteristiska och som bidrar till cancerbildning. Dessa områden utgörs av olika signalvägar eller proteiner som är defekta och som kan ge upphov till cancer. För paper III och paper IV var fokus främst det område som handlar om cancercellens förmåga att undvika att dö dvs att undgå det som kallas cellens självmordsprogram.

Cancercellens självmordsprogram påverkas av PKC

Självmordsprogrammet ska enbart starta då en cell är så pass skadad, t ex av gifter eller mutationer, att den inte kan fungera korrekt. Regleringen av självmordsprogrammet sköts av olika proteiner. I paper III tittade vi närmre på Smac, ett av de proteiner som reglerar cellens självmordsprogram. Vid cellskada bidrar Smac till celldöd genom att hindra funktionen av ett annat protein, IAP, som annars kan bromsa eller stoppa celldöd. Vi såg att PKC-familjemedlemmen PKC δ kunde binda till Smac. Då vi aktiverade PKC kunde denna bindning stärkas och motverka celldöd av paclitaxel, ett cancergift.

I paper IV fortsatte vi studera celldöd genom att använda en kemisk framställd molekyl som ska likna en del av proteinet Smac. Dessa kallas därför "Smac mimetics" vilket betyder Smac-härmare. Den Smac-härmare vi använt kallas LBW242.

LBW242 är tänkt att fungera genom att hindra det celldödsbromsande proteinet IAP och på så sätt underlätta för celldöd i cancerceller. LBW242 har dock ytterligare egenskaper. LBW242 och andra Smac-härmare kan påverka signaleringen från en receptor. En receptor är en mottagare och vidarebefordrare av signaler som sitter på cellens yta. Då LBW242 tillsätts ändrar receptorn sitt sätt att signalera i vissa celler. Vissa cancerceller ändrar om till ett "celldödsläge" och cancercellen dör. Vissa cancerceller svarar dock inte med detta "dödsläge" utan är okänsliga för LBW242. I paper IV visar vi att då man kombinerar LBW242 med ett ämne som aktiverar PKC (TPA) så dör en viss grupp bröstcancerceller. Dessa bröstcancerceller tillhör en grupp som man brukar klassificera som svårbehandlade.

I denna avhandling har vi sett att PKC α kan vara en markör för vissa aggressiva bröstcancer. Vidare har vi sett att PKC α kan styra regleringen av stanniocalcin-1.

Vi har även sett att PKC δ binder till Smac och att en intakt bindning gör cellen mindre känslig mot ett särskilt cancergift. Utöver detta ser vi att PKC-aktivering tillsammans med en Smac-härmare leder till celldöd i vissa bröstcancerceller.

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My mind has gone loose inside its shell
The National

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