Relevance of Atypical Protein Kinase C Isotypes to the Drug Discovery Process

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Concerted Protein Phosphorylation Events Mediated by Atypical PKC Isotypes

Protein phosphorylation regulates most aspects of cell life in a hierarchically ordered sequence, and dysfunction of concerted phosphorylation is thus associated with various facets of neoplasia, including proliferation, survival, invasion, angiogenesis and metastasis. The activation of phosphorylation cascades is generally triggered by specific agonists at the plasma membrane by means of signalling cascades that ultimately control specific effectors such as small GTPases, mitogen-activated kinases, cell-cycle-controlling proteins and transcription factors. Much of the regulation of these pathways is achieved by modulation of the phosphorylation status of various components of these cascades. The protein kinase C (PKC) family of serine/threonine protein kinases plays important and diverse roles within these signal-transduction pathways, in mediating the effects of many extracellular stimuli including growth factors, hormones and drugs.[1] Atypical protein kinase C isoenzymes (aPKCs) are part of these complex signal-transduction networks with a "master switch" function in balancing between cell proliferation and programmed cell death and in controlling cellular architecture and polarity. Extensive efforts to develop aPKC-specific inhibitors have been undertaken, with the rationale that they might counteract acquired capabilities of cancer cells. The criteria that qualify a particular kinase as a putative drug target are, however, not straightforward. We shall therefore discuss some structural and functional aspects of aPKCs with relevance to the drug-discovery process.

Structural Characteristics of Atypical PKC Isotype Members of a Comprehensive Protein Family

Protein kinase C isoenzymes represent a family of structurally related serine/threonine protein kinases known to comprise 11 isotypes (Figure 1).[2] The various PKC isoforms are classified by structural similarities and cofactor requirements into three major subgroups: i) the calcium- and DAG-dependent (DAG = diacylglycerol) classical or conventional PKC isoforms (cPKCs) cPKC-α, cPKC-βI, cPKC-βII and cPKC-γ, ii) the calcium-independent but DAG-responsive PKC isotypes, which have been termed novel PKCs (nPKCs) and comprise the isoenzymes nPKC-δ, nPKC-ε, nPKC-η and nPKC-θ, and iii) the so-called atypical PKC isoforms aPKC-ζ and aPKC-ι (or its mouse homologue -λ), which differ from other PKCs in their insensitivity to calcium, DAG and phorbol ester.[3,4] Recently, an isotype with a novel atypical aPKC-ζ catalytic domain, the so-called PKM-ζ, was described.[5] This novel family member is a kinase synthesised in an autonomously active form, and its activity is both necessary and sufficient for the maintenance of hippocampal long-term potentiation (LPT) and the persistence of memory in *Drosophila*. Recently, Parkinson and co-workers have identified another new member of the aPKC family—designated aPKC-ζII—functionally involved in cell polarity through inhibition of tight junction formation.[6]

If the structural aspects of PKC isoenzymes are compared, it is clear that atypical PKCs differ significantly from other family members not only in the regulatory domains, which lack functional domains binding diacylglycerol, phorbol ester or calcium, but also in their catalytic domains comprising the ATP- and substrate-binding domains (Figure 1). The aPKC regulatory domain includes the pseudosubstrate (PS) sequence, the PB1 domain (containing the so-called OPCA motif)—unique within PKC isoenzymes—and an atypical C1 domain of a single Cys_2His_2-rich zinc finger motif. With these structural differences in their regulatory domains, aPKC isoforms require distinctly different cofactors for their activation. These isoenzymes exhibit elevated basal enzyme activity but, instead of calcium and DAG, there are several adaptor proteins, such as λ-interacting protein (LIP) and p62/ζ-interacting proteins (ZIP1 and ZIP2), that enhance enzymatic activity.[7] The interacting proteins ZIP1 and ZIP2 are thought to play pivotal roles in scaffold signalling, such as phosphatases and ion channel proteins.[8] Furthermore, inhibiting proteins have also been identified. The most prominent protein to interfere with aPKC function is the prostate apoptosis-response protein Par-4.[9] An autoinhibitory mechanism is manifest through the PS sequence, a short stretch of amino acids resembling a substrate motif that occupies the substrate-binding cavity. In the absence of a stimulus, the catalytic domain binds to the pseudosubstrate domain; this causes the enzyme to fold above the hinge region linking the C2 and C3 regions and results in suppression of kinase activity. Like other members of the AGC group of protein kinases, atypical PKC isotypes possess an ATP-binding pocket, an activation loop motif and a hydrophobic stretch. As far as the ATP-binding pocket is concerned, the most conserved and probably

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best characterised residue of protein kinases is the so-called *invariant lysine* in subdomain II of the catalytic domain, corresponding to Lys274 in human aPKC-ι and Lys281 in aPKC-ζ. This *invariant lysine* is directly involved in the phosphotransfer reaction and interacts with the α- and β-phosphates of ATP, thereby anchoring and orienting the nucleotides. Replacement of the invariant lysine by any other amino acid, including arginine, generally leads to loss of catalytic activity, and site-directed mutagenesis of this residue has become almost a standard approach by which to generate dominant negative, kinase-dead mutants, as has successfully been shown for aPKC-ζ, nPKC-θ, cPKC-α and nPKC-ε.[10] In a generally accepted activation model, Thr410 and Thr560 in the activation loop and turn motif play a pivotal role for primed activation. For full activation, however, additional cofactors such as phosphatidylinositol, phosphatidic acid, arachidonic acid or ceramide are required.[11] Although PI 3,4,5-trisphosphate (PIP3) is thought to be solely responsible for enhancing the activity of PDK-1, a direct activating effect of PIP3 through a phosphorylation-independent but conformation-dependent relief of pseudosubstrate autoinhibition has also been suggested.[12] This activation model of aPKC does not seem to be complete, since other findings have shown aPKCs to be active even in the absence of functional PI3-kinase.[13] We have demonstrated that atypical PKC-ι is enzymatically controlled through a direct phosphorylation event on Tyr421 via the ATM/c-Abl pathway routing DNA damage, cell cycle arrest and survival.

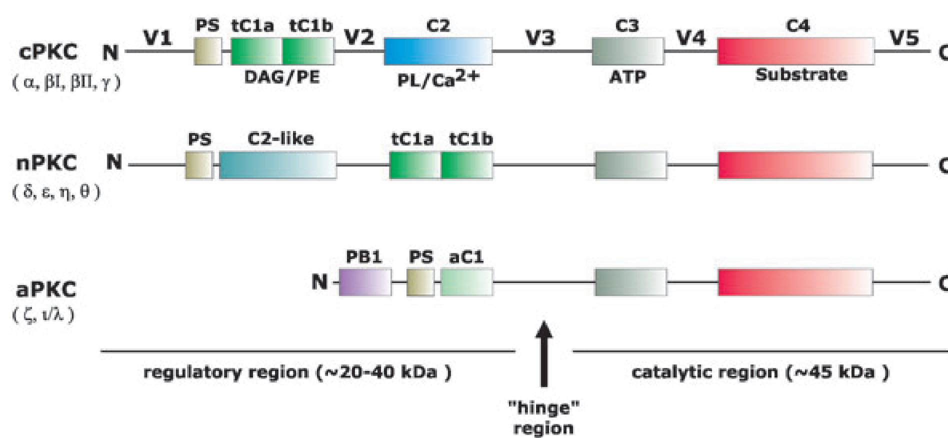

Figure 1. Structures of PKC family members. Protein kinase C isoenzymes possess regions that are highly conserved between different PKC isozymes (regions C1 to C4) and variable regions (regions V1 to V5). The N-terminal regulatory region possesses an autoinhibitory or pseudosubstrate domain (PS) and a C2 domain involved in the binding of the phospholipid (PL) cofactors, Ca^{2+} and proteins that regulate activity and localisation of classical and novel PKCs. Phorbol esters (PEs) and the second messenger diacylglycerol (DAG) bind to the cysteine-rich motif (tC1a/tC1b; typical C1 region a and b) present in cPKCs and nPKCs, whereas aPKCs harbour an atypical C1 region (aC1) insensitive to PE and DAG. Furthermore, the PB1 (Phox and Bem1) domain, only found in aPKCs, contains the OPCA motif mediating protein–protein interactions. The C-terminal catalytic region includes the motifs involved in binding of substrate (C4 region) and of ATP (C3 region), the latter harbouring furthermore an "atypical" sequence motif (for details please refer to Figure 2). The numbers of amino acids in the PKC family members vary according to the subclasses, c- and n-PKC having 672–737 amino acids, and the atypical PKCs 587 (aPKCι) or 592 (aPKCζ).

The assumption that tyrosine-dependent enzymatic regulation of aPKCs is fundamental for cellular function is further supported by the fact that NGF-mediated (nerve growth factor) tyrosine phosphorylation at the lip of the activation loop (Tyr256) is a critical step in nuclear import of activated aPKC-ζ.[14] As far as protein degradation of aPKC isotypes is concerned, it should be mentioned that von Hippel–Lindau tumour suppressor has been reported to mediate ubiquitination of activated aPKC-λ/ι by undocking via the target recognition β-domain at the regulatory domain of the kinase.[15]

The Catalytic Domain of aPKCs—A Putative Pharmaceutical Intervention Motif

The catalytic domain is characteristic of all protein kinases, with a remarkable degree of sequence identity between the over 180 known mammalian kinases, and highly conserved from yeast to man. The 200–300 amino acid residues of the catalytic core region are predicted to fold into a common 3D structure, as revealed by crystal-structure analyses of several protein kinases.[16] Some protein kinase motifs are extremely well conserved. One of these motifs is the glycine-rich loop with the consensus sequence GXGXXG located in the first of 12 subdomains of a protein kinase catalytic domain. The three glycine residues of the glycine-rich loop are conserved in over 95% of the human kinases known so far and even in other nucleotide-binding proteins, such as the small G proteins Ras, Rac and Rho. They fold into a β-strand–turn–β-strand structure and form a flexible clamp that covers and anchors the nontransferable phosphates of ATP.[17] The glycine residues provide the flexibility necessary for anchoring the ATP molecule and excluding the water. For this reason they are essential both for effective catalytic activity and for low ATPase activity because of phosphate transfer to water molecules.

Apart from the differences in the regulatory domains, the catalytic domains of aPKCs differ considerably from those of other known kinases, as they each contain an alanine residue instead of the third conserved glycine: Gly-Gly-Ala within the glycine loop motif (for details see Figure 2). We have used site-directed mutagenesis and kinetic analysis to investigate whether these sequence differences affect the nucleotide-binding properties and catalytic activity of aPKC-ι.[18] When Lys274, a residue essential for ATP binding and activity, conserved in most protein kinases, was replaced by arginine (K274R mutant), aPKC-ι retained its normal kinase activity. This is in sharp contrast to all other PKCs or even distantly related kinases such as phosphoinositide 3-kinase γ (PI3Kγ), in which the same mutation completely aborted the kinase activity. In addition, the sensitivity of aPKC-ι to inhibition by GF109203X, a substance acting on the ATP-binding site, was not altered in

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PKC-βI   LMVLGKGSFGKVMLS ERKGTDELYAVKILK KDVVIQDDDVECTMV EKRVLAL  396
PKC-βII  LMVLGKGSFGKVMLS ERKGTDELYAVKILK KDVVIQDDDVECTMV EKRVLAL  396
PKC-α    LMVLGKGSFGKVMLA DRKGTEELYAIKILK KDVVIQDDDVECTMV EKRVLAL  393
PKC-γ    LMVLGKGSFGKVMLA ERRGSDELYAIKILK KDVIVQDDDVDCTLV EKRVLAL  405

PKC-δ    HKVLGKGSFGKVLLG ELKGRGEYFAIKALK KDVVLIDDDVECTMV EKRVLTL  403
PKC-θ    HKMLGKGSFGKVFLA EFKKTNQFFAIKALK KDVVLMDDDVECTMV EKRVLSL  434
PKC-ε    IKVLGKGSFGKVMLA ELKGKDEVYAVKVLK KDVILQDDDVDCTMT EKRILAL  462
PKC-η    IRVLGKGSFGKVMLA RVKETGDLYAVKVLK KDVILLDDDVECTMT EKRILSL  406

PKC-ζ    IRVIGRGSYAKVLLV RLKKNDQIYAMKVVK KELVHDDEDIDWVQT EKHVFEQ  298
PKC-ι    LRVIGRGSYAKVLLV RLKKTDRIYAMKVVK KELVNDDEDIDWVQT EKHVFEQ  299
             WW ATP                    WW ATP
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Figure 2. Sequence alignment of the ATP-binding domains (C3 region) of human PKC family members. The ATP-binding domains of atypical PKCs differ considerably from those of other PKC isoenzymes, as they each contain an alanine residue instead of the third conserved glycine in the glycine loop motif.

the K274R mutant. In contrast, replacement of Lys274 by tryptophan (K274W) completely abolished the kinase activity of aPKC-ι. This defect could not be reversed by cotransfection of λ-interacting protein (LIP), a protein activator of aPKC-ι that is able to stimulate in vitro activity of aPKC-ι extracted from co-transfected COS cells. In agreement with results obtained with other kinase-defective PKC mutants, in cultured cells, aPKC-ι K274W operated in a dominant negative fashion on signal-transduction pathways involving endogenous aPKC-ι, while the effect of the catalytically active K274R mutant was identical to that of the wild-type enzyme. In brief, aPKC-ι also differs from classical and novel PKCs in the catalytic domain, representing a new aspect of an ATP-binding mechanism and probably catalysis. The structural difference is correlated with an unusual catalytic activity only known from a small number of distantly related kinases such as CDK-activating kinase (Cak) from yeast. These results further suggest that aPKCs possess a unique ATP-binding domain. Despite the fact that the ATP-binding site in the catalytic domain belongs to the most specific sequences within the protein kinase family, the most specific inhibitors of PKC bind to this part of the molecule and act by blocking ATP binding to the enzyme. This is the case for the bisindolylmaleimide GF109203X and other homologues of the potent PKC inhibitor staurosporine. It is intriguing that even within the PKC family these inhibitors exhibit clear isoenzyme specificity, affecting atypical isoforms more than 100 times less powerfully than classical or novel PKCs.[10b] This, together with the remarkable difference within the glycine-rich loop in relation to other kinases, suggests that aPKCs, surprisingly, differ significantly from other PKC isoforms and protein kinases in general in the structures of their catalytic domains. From a pharmaceutical point of view, this knowledge should prove useful in the development of specific inhibitors of atypical PKC isotypes.

The OPCA Motif Novel Regulatory Domain Characteristics as Targets for Intervention

Atypical PKC isotypes can be distinguished from the other family members by their recently identified PB1 (Phox and Bem1) domains in their N termini, which offer them novel interaction avenues. Ito and co-workers demonstrated that the PB1 domain recognises Phox and Cdc24p (PC) motifs for functional protein–protein interaction,[19] and there is no doubt that the PB1 domain harbours different motifs (OPR/PC/AID; **O**cticosa **P**eptide **R**epeat/**P**hox and **C**dc/**A**typical PKC **I**nteraction **D**omain) for fine-tuning of cellular processes. Ponting and co-authors have therefore suggested the renaming of the motif to OPCA in order to make it clear that the OPR/PC/AID motifs are localised within a larger PB1 domain.[20] Generally, PB1 domains can be divided into three classes: the A, B and AB types. An A-type PB1 domain (cdc24, p40phox) contains an OPCA motif, whereas a B-type PB1 domain (Bem1p, p67phox) lacks this motif but shows a signature of basic amino acid residues. AB-type PB1 domains tend to form front-to-back arrays (aPKCs, p62/ZIP, Par6).[21] Structural NMR analysis of the Bem1p PB1 domain has revealed that it is composed of two α helices with a four-strand mixed β sheet. The inner β1 and β4 strands show parallel orientation, and β2 and β3 an antiparallel orientation relative to β1 and β4. This structural property can be regarded as a ubiquitin-like β-grasp fold.[22] Comparison of the Bem PB1 primary sequence with other PB1 proteins containing OPCA motifs shows high sequence conservation in the functional hydrophobic core. The OPCA motif is made up of about 20 acidic and hydrophobic amino acids with the consensus sequence *XYXDEDGDX*-X*XSDED/E*X, where * corresponds to a hydrophobic amino acid and X to any amino acid.[23] Interestingly, the OPCA motif is not present in all PB1 proteins, it having recently been determined that for PB1 heterodimerisation only one interaction partner has to contain an OPCA motif.[21] Currently, about 13 mammalian PB1-containing proteins have been identified (SMART domain data base). According to the varying domain compositions, these candidates can be further categorised into kinases (K) or adaptor and scaffold (AS) proteins. Among them are the atypical PKCs (K), MEKK2/3 (K), MEK5 (K), p62/ZIP (AS), the Par6 family (AS), p40phox and p67phox (AS), TFG (TRK fused gene) (AS) and NBR1 (next to breast cancer 1) (AS).[24]

The aPKC–OPCA Par6 Connection

Atypical PKC-ι/λ and -ζ have been shown to interact directly with Par6α (**Par**titioning defective **6α**).[25] Substitution of the Par6α lysine residue 19 by alanine (K19A) completely disrupted the aPKC (both ι/λ and ζ) interaction with Par6α. Alanine scanning mutational studies of other conserved amino acid residues did not affect the aPKC–Par6α interaction (Asp63, Asp67, Leu69, Ser76). On the other hand, substitution of Lys20 (K20A) in aPKC-ι/λ or Lys19 (K19A) in aPKC-ζ did not affect aPKC–Par6α dimerisation.[26] Conserved aspartate residues seem to

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be critical for OPCA-mediated interaction with Par6α. Exchange of Asp63 (D63A) in aPKC-ι/λ or Asp62 or -66 (D62A, D66A) in aPKC-ζ led to the disruption of the interaction.[27] These findings suggest an essential role of Lys19 in Par6α in interaction with aPKCs. Additionally, the aspartate residues 63 (aPKC-ι/λ) and 62/66 (aPKC-ζ) seem to be critical for the interaction. These results suggest that the interaction between aPKCs and Par6 is mediated by the Par6 lysine residue. Currently two main consequences of the aPKC-Par6 interaction have been described in the literature: i) the complex plays an important role in regulating polarity decisions of epithelial cells,[28] and ii) Par6 is involved in the regulation of insulin-dependent glycogen synthesis through aPKC-mediated phosphorylation events.[29]

The aPKC–OPCA ZIP Connection

There is a growing body of evidence that p62/ζ-interacting protein/aPKC-ζ interactions occur in a manner similar to that discussed above for Par6; p62/ZIP also harbours a functional OPCA motif with the corresponding lysine residue. Mutational studies have shown that ZIP is able to bind aPKC-ι/λ K20A, but not the corresponding D63A mutant. Alanine K7A substitution in ZIP completely disrupted the interaction with aPKC-ι/λ. These findings are in agreement with a tentative model in which the OPCA motif of aPKCs interacts with the conserved lysine of PB1 interaction targets.[26] In other words, aPKC-ζ interacts through its acidic front with the basic back of ZIP/p62 and Par6.[24] Experiments on employment of RNAi technology should further verify the assumption that small peptides that interfere with the corresponding lysine block ZIP-mediated kinase activity and protein scaffolding with relevant effector molecules such as phosphatases and ion channel proteins.

The aPKC–OPCA MEK5 Connection

Erk5 has recently been demonstrated to be the newest subfamily member of the MAPK family, playing an integral function in the transcriptional activation of c-Fos, Fra-1 and cyclin D1,[30] and aPKCs have been shown to be partially involved in signal transduction by ERK1/2 family members.[31] Diaz-Meco et al. have recently described MEK5 as a new target of atypical PKC isotypes in mitogenic signal transduction. In more detail, the AID sequence of the OPCA motif was determined to be a surface of interaction between MEK5 and aPKC-ζ. Consistent with this, OPCA deletion mutants of MEK5 interact weakly with aPKC-ζ, and it has also been shown that over-expression of the aPKC-ζ V1 domain is sufficient for interaction with MEK5, while EGF stimulation strengthens the interaction of aPKC-ζ with MEK5. As a prerequisite for EGF-mediated signal transmission, MEK5 has to be activated by a MEKK.[32] Over-expression of the MEK5-OPCA fragment overrides aPKC-ζ MEK5 interaction in a competitive manner. Interestingly, MEK5 lacks the conserved lysine residue but contains the OPCA motif. It is assumed that the aPKC–MEK5 interaction is mediated by aPKC isoenzymes conserved lysine residue, because aPKC-ι/λ K20A mutants are incapable of binding MEK5, while the MEK5 interaction remains unaffected with aPKC-ι/λ D63A mutants.[26] In contrast to Diaz-Meco et al., Lamark and co-workers do not propose a strong direct interaction between aPKCs and MEK5,[24] but favour a tentative model in which p62/ZIP further stabilises a weak interaction of aPKCs with MEK5. Coexpression studies suggest that MEK5/aPKC colocalisation takes place only when both partners are coexpressed at approximately equal levels. Interestingly, the finding that aPKC-ζ interacts with MEK5—through the OPCA motif—could be the missing link in understanding the mode of action in transcriptional activation of cyclin D1 through the ERK5 cascade. Previously we had demonstrated that transcriptional activation of cyclin D1 by transformation of Ras is Rac-dependent and requires the PKC isotypes nPKC-ε and aPKC-λ/ι and -ζ, but not cPKC-α. We were not able, however, to demonstrate a direct physical interaction of MEK1 with aPKC-ζ. Consistent with the findings of Mulloy and co-workers, explaining the dependency of MEK5/ERK5 cascade for cyclin D1 transactivation, we would like to propose that an alternative pathway via Ras-MEK5/aPKC/OPCA (Ras-MEK5-p62/ZIP-aPKC) to the cyclin D1 promoter might exist. Together with findings from others,[33] explaining the absolute dependence on cyclin D1 in Neu- and Ras-mediated malignant transformation in mammary epithelial cells, intervention with PKC isotype-specific inhibitors interfering with the OPCA motif might be a promising therapeutic approach. The fact that MEK5 has been recognised as a key signalling molecule associated with prostate carcinogenesis is a further indication of the pivotal role of the aPKC–OPCA MEK5 connection in cancer development.[34]

Are There Any Relevant Expression Profiles of Atypical PKC Isotypes in Tumour Cells?

Isoenzyme expression patterns of classic and novel PKCs are well documented for several carcinomas, but only sparse data relating to the expression profiles of atypical PKC isotypes are available. Cornford et al. have reported aPKC-ι to be widely expressed in both benign and malignant prostatic epithelial cells. No significant elevation in aPKC-ι expression could be detected in malignant versus benign prostatic specimens, whereas a marked increase in aPKC-ζ expression in prostate cancer cells relative to benign control tissues was observed.[35] Since aPKC-ι has been reported to protect other types of neoplastic cells from drug-induced apoptosis,[36] the overall expression of aPKC-ι might explain the usually low rates of apoptosis in benign and malignant prostatic cells.[37] The involvement of aPKC-ζ in critical survival pathways mentioned below, together with the high expression level of this isotype in prostate cancer cells, might account for the high incidence of resistance of these cells to chemotherapy. Furthermore, over-expression of aPKC-ζ has been reported in carcinomas of liver and urinary bladder.[35,38] Weichert et al. recently reported that normal ovarian-surface epithelium, benign cystadenomas and borderline tumours did not express aPKC-ι, whereas it was strongly expressed in a subset of ovarian carcinomas. In this context, a significant positive correlation for aPKC-ι expression with the

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clinicopathological factors, Silverberg grading and FIGO-stage could be established.[39]

Atypical PKC Isotypes under the Influence of the Ha-ras Oncogene

Apoptosis and cell-cycle progression are tightly controlled and interconnected processes that ensure genetic fidelity during cell proliferation. Oncogenic Ras has been reported to play a pivotal role in these processes, for which atypical PKC isotypes are thought to be functional prerequisites. The Raf/MEK/ERK pathway is believed to be a major route for mitogenic actions downstream of Ras. Raf, a well known Ras-interacting partner, phosphorylates MEK, which in turn phosphorylates and activates ERK, a protein kinase that translocates to the nucleus and activates transcription factors required for cell-cycle progression.[40] On the other hand, oncogenic Ras activates PI-3-kinase, which produces PIP_3 and thereby activates PDK-1, which in turn modulates the phosphorylation state of a critical residue in the aPKC activation loop.[41] Several studies have reported that aPKCs are able to activate MEK and ERK in vivo and in vitro.[42] For this reason, one can speculate that the signals from Ras affecting Raf and the aPKCs meet at the level of MEK. By employing a combination of mRNA antisense constructs, kinase-defective, dominant negative and constitutively active PKC mutants, we have provided evidence that the transformation of Ras employs three PKC isozymes that operate in a hierarchically ordered sequence for the transcriptional activation of c-fos. Two of them, nPKC-ε and aPKC-ζ, have been shown to act downstream of Raf-1 and MEK; this suggests that PKC isotypes might be involved in: i) the activation of ERKs, ii) the control of duration and extent of the active state, iii) the regulation of the interaction with scaffold proteins such as MP-1 and iv) a process required for translocation of dimerised ERKs from the cytosol to the nucleus. We have shown that transcriptional activation of cyclin D1 through the transformation of Ha-Ras is MEK- and Rac-dependent and requires the PKC isotypes -ε, -λ/ι and -ζ, but not cPKC-α.[31a] In this context, evidence has been presented that aPKC-λ/ι acts upstream of Rac, between Ras and Rac, whereas the PKC isotypes -ε and -ζ act downstream of Rac and are required for the activation of ERKs. Ras signalling also affects the small GTPases CDC42 and Rac, which play critical roles in the reorganisation of the actin cytoskeleton, an essential feature of the morphological changes associated with the induction of the transformed phenotype.[40] Work from our lab provided evidence that aPKCs are required for actin remodelling mediated by Ras downstream of either Rac or CDC42.[10b] Furthermore, we have demonstrated that the reorganisation of the actin cytoskeleton induced by oncogenic Ras is mediated by two atypical PKC isozymes, whereas aPKC-λ/ι acts upstream of PI-3-kinase and Rac-1, while aPKC-ζ is localised downstream of Rac but upstream of Rho. In summary, these data support the concept that oncogenic Ras mediates and requires the coordinated activation of Rac and Rho and further that PKC isozymes are involved in this mechanism. Another effect of Ras signalling involves the activation of NFκB, which is required for cell survival and for Ras-induced transformation.[43] Atypical PKCs are generally believed predominantly to exert an antiapoptotic function by promoting cell survival and proliferation. The antiapoptotic effect of aPKC family members has been ascribed to activation of NFκB, which may be mediated through their regulatory function in ERK activation.[44] Combination treatment of cells with PKC inhibitors has been shown to enhance the antitumour activity of several chemotherapeutic drugs.[45] In particular, atypical PKC members have been shown to confer resistance to numerous apoptosis-inducing stimuli, such as irradiation and chemicals (IR,[46] UV,[47] etoposide,[48] taxol[13] and ocadaic acid,[36] as well as the physiological ligands tumor necrosis factor α (TNFα)[11c] and nerve growth factor (NGF)[49]). In the TNFα signalling pathway, aPKC-ζ takes a central position in mediating NFκB activation.[7c,d] Upon stimulation, TNF receptor 1 interacts with the death-domain kinase RIP through the adapter molecule TRADD. RIP recruits aPKCs through the p62/ζ-interacting protein (ZIP), and aPKCs in turn associate with and directly phosphorylate IκB kinase (IKK), leading to IκB phosphorylation and degradation and thereby to nuclear translocation of NFκB.[50,7d] These events correlate with an accumulation of the caspase inhibitory protein XIAP, expression of which was shown to be regulated by NFκB.[51] Another apoptosis-modulating NFκB-independent pathway involves the p62/ZIP-mediated targeting of aPKC-ζ activity to the Kvβ2 subunits of potassium channels, which exert regulating functions in some apoptotic pathways.[52]

Furthermore, aPKC-ζ was reported to modulate the apoptotic machinery both at the mitochondrial level, by modifiying the Bcl-2/Bax ratio,[53] and at the postmitochondrial level, by modulating cytochrome c-mediated caspase activation,[54] but the mechanisms by which PKC regulates these effectors are still unclear.[48]

Routing DNA Damage by Interfering with the ATM-c-Abl-aPKC Pathway

DNA strand breaks—induced by oxidative stress, ionising radiation, chemotherapeutic agents or UV light—result in the binding and activation of serine/threonine kinases of the phosphoinositide-3-kinase family. Members of this kinase family are ATM (*A*taxia-*T*elangiectasia, *M*utated), ATR (*A*taxia-*T*elangiectasia and *R*ad3-related) and the *DNA*-dependent *p*rotein *k*inase (DNA-PK). These proteins are activated by direct binding to the site of DNA damage. Depending on the severity of DNA damage, this leads either to cell-cycle arrest and activation of DNA-repair mechanisms or to induction of apoptosis. Although a variety of substrates of these types of kinases have been identified, including prominent regulators of cell-cycle arrest and apoptosis such as Chk2 and p53, the central effector seems to be the Src-related tyrosine kinase c-Abl. ATM directly activates c-Abl by serine phosphorylation in the kinase domain,[55] and c-Abl in turn phosphorylates tyrosine residues and activates proapoptotic proteins such as p53, p73, JNK/SAPK and nPKC-δ.[56] However, despite this central role in the induction of cell cycle arrest and apoptosis, the constitutively active Abl mutants v-Abl and Bcr-Abl were originally identified as the oncogenes responsible for certain forms of murine leu-

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kaemia and human chronic myelogenous leukaemia (CML), respectively. Surprisingly, the resulting tumours are highly resistant to treatment with DNA-damaging agents known to activate c-Abl. This may in part be explained by a delay in the G2/M phase of the cell cycle, which gives the cell a chance to complete DNA repair, replication and chromosomal segregation, thereby preventing a mitotic catastrophe.[57] However, Bcr-Abl also activates signalling molecules that inhibit the apoptotic machinery, such as Stat5, Ras, Bcl-x_L or the atypical aPKC-ι. Among these, aPKC-ι seems to be of special importance, as inhibition of aPKC-ι can completely reverse the resistant phenotype mediated by Bcr-Abl.[58,13] As the constitutive tyrosine kinase activity of Bcr-Abl is caused by fusion of the Abl catalytic domain to the Bcr protein and not by mutation of the primary sequence, it is unlikely that its substrate specificity is fundamentally different from that of c-Abl. Recently we have demonstrated that aPKC-ι is a direct target of c-Abl.[59] It can be deduced from the homology to the PKA catalytic domain that, in the 3D structure, the relevant tyrosine lies in close proximity to the phosphoinositide-dependent kinase 1 (PDK-1) phosphorylation site Thr403 in the activation loop (for details see Figure 3), which is required for *priming* of PKCs, enabling them to be activated by other stimuli. Thus, we assume that Tyr421 phosphorylation has an effect similar to activation loop phosphorylation, resulting in enhanced substrate binding and a faster chemical catalysis step. Our data, which show that aPKC-ι activation by cAbl is mediated by direct binding and subsequent tyrosine phosphorylation, are consistent with the results of Jamieson et al., who described a PI3K-independent activation of aPKC-ι by oncogenic Bcr-Abl.[13] However, this observation does not rule out a contribution of PDK phosphorylation for aPKC-ι-mediated drug resistance. As PKC phosphorylation at the activation loop seems to be a priming step that makes the enzyme sensitive to other activators, it might increase the susceptibility of aPKC-ι for activation by c-Abl. Interestingly, it was shown in a recent study that ionising radiation can induce nuclear translocation of atypical aPKC-ζ, and that this translocation was sensitive to the PI3K inhibitor wortmannin.[46] This means that PDK could provide two prerequisites for aPKC activation after DNA damage: i) activation loop phosphorylation as a priming step for enzymatic activity, and ii) translocation to the nucleus, where the aPKC is activated by c-Abl tyrosine phosphorylation. Thus, aPKC-ι is involved in a pro-survival signalling pathway initiated by c-Abl following DNA damage. Given the central role of c-Abl for the sensitivity to DNA damaging agents, these observations may contribute to a better understanding of this cellular response, and might prove helpful for overcoming resistance to chemotherapeutic agents. In summary, as in the response to activation of the TNF-α receptor, the putative potency of aPKCs to shift the equilibrium of intercellular signals from induction of cell death to cell survival is part of a model in which inhibition of aPKC partially overcomes stress- and drug-induced chemoresistance.

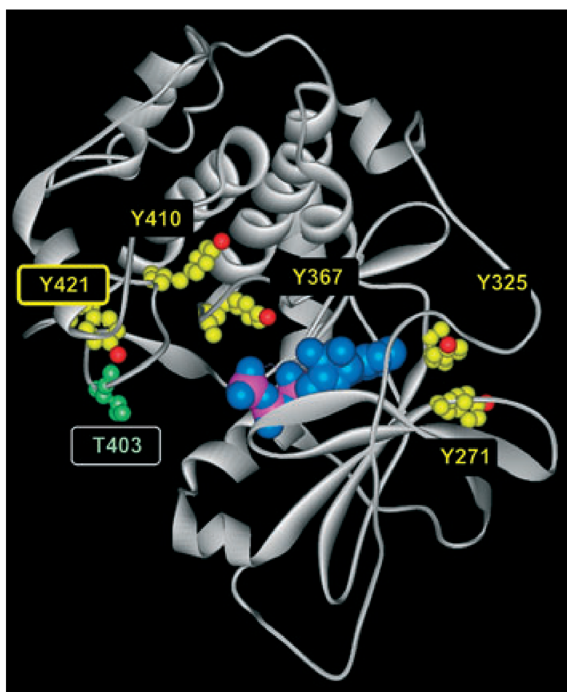

Figure 3. 3D representation of the position of the tyrosine phosphorylation site within the catalytic domain. The structure of the homologous catalytic domain of PKA, as deposited in the PDB database (PDB ID: 1CDK) by Bossemeyer et al.,[65] has been modified by Martin Spitaler to show the position of the tyrosines (Y) corresponding to the potential phosphorylation sites in aPKC-ι, and the threonine (T) phosphorylated by PDK. The numbers correspond to the amino acid positions in human aPKC-ι. Figure reproduced with kind permission from EMBO Journal.

Inhibition of Atypical PKC Isotypes Sensitises Tumour Cells to Widely Used Chemotherapeutic Agents

Activation of survival pathways by disregulation of apoptosis through superimposed kinases is of critical importance for malignant transformation and maintenance of the transformed phenotype. PKC isotypes cPKC-α, nPKC-θ and aPKC-ζ, as well as the most prominent BAD kinase PKB/Akt, have frequently been associated with such mechanisms. Recently, we showed that fully activated aPKC isozymes ι and ζ protect cells from programmed cell death after expression of oncogenic Ras, inhibition of cell attachment and treatment with cisplatin or camptothecin.[59] The mechanisms by which these kinases are activated under these conditions are incompletely understood. Ras had earlier been shown to activate the phospholipase D (PLD) isozymes PLD1b and PLD2, and evidence has been presented that PLD1b acts upstream of PKC, which in turn activates PLD2.[60] It has been suggested that the data indicate a putative signalling cascade comprising Ras→RalA→PLD1b→aPKC-ι→PLD2. Over-expression of a constitutively active mutant of aPKC-ι protected cells from camptothecin (Figure 4A/B) and exposure to camptothecin activated aPKC-ι (Figure 4C). Camptothecin, like other DNA-damaging agents, is known to activate c-Abl by the ATM connecting pathway. To verify that this camptothecin-induced activation of aPKC-ι is mediated by cellular Abl, we used the Abl inhibitor STI571 (Imatinib). As shown in Figure 4C, enzymatic activation of aPKC-ι by camptothecin was partially inhibited in the presence of Imatinib in a dose-dependent manner. This result is part of a

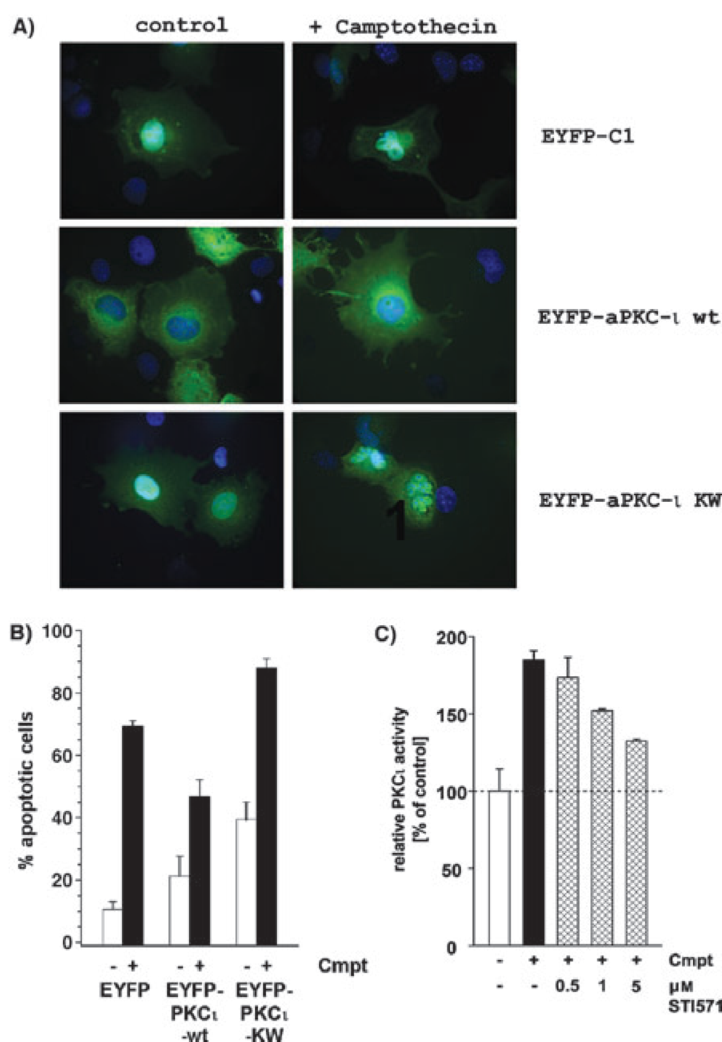

Figure 4. Rescue of camptothecin-induced cell death by active aPKC-ι. Prostate carcinoma (PC3) cells grown on glass cover slips were transiently transfected with EYFP-C1, EYFP-aPKC-ι-wt or kinase-inactive EYFP-aPKC-ι-KW. After 24 h, cells were either left untreated (0.5 % DMSO control) or were treated with camptothecin (Cmpt; 10 μM) for an additional 24 h. Thereafter, the cells were fixed, the nuclei were stained with DAPI, and the nuclear fragmentation of green, EYFP-positive cells was observed by fluorescence microscopy. A) Nuclear fragmentation in EYFP-expressing PC3 cells. EYFP is shown in green, nuclear DAPI staining in blue. The cells shown are representative of three independent experiments. B) Quantification of the results: the number of cells with nuclear fragmentation was determined by observation of 50 cells per coverslip in triplicate. The data bars show the mean of three independent experiments ±SEM ($p < 0.02$). For comparison of grouped data, Student's t-test was applied. P values below 0.05 were considered to indicate significant differences. C) Activation of aPKC-ι by addition of camptothecin (10 μM) to the cells 1 hour before extraction and inhibition of this effect by preincubation with the cAbl inhibitor STI571 (Imatinib). COS-1 cells were transiently transfected with plasmids encoding RGS-His_6-tagged PKCι 48 h after transfection, PKCι was purified with Ni^{2+}-NTA agarose and its enzymatic activity was measured in vitro. To compensate for variable transfection efficiencies, the results were standardised by cotransfection of a plasmid encoding firefly luciferase under the control of a constitutive SV40 promoter and measurement of luciferase in an aliquot of the lysates. Data shown are means of at least six parallel measurements from two independent experiments ±SEM ($p < 0.03$).

tentative model in which camptothecin-induced DNA damage influences aPKC-ι activation in a c-Abl-dependent manner. Treatment of cells with cisplatin (cis-DDP) also has the ability to activate aPKC-ζ. Expression of a kinase-defective, dominant negative aPKC-ζ sensitises cells to cis-DDP, indicating a protective effect of wild-type aPKC-ζ on cis-DDP-induced cell death.[59] Evidence was presented that aPKC-ζ-mediated phosphorylation of pro-apoptotic Bad may contribute to the anti-apoptotic effect of aPKC-ζ. Atypical PKC-ζ phosphorylates Bad at the critical serine residues: Ser112 and Ser136. Ser112 phosphorylation has been shown to be mediated by the Raf-ERK pathway, whereas protein kinase B/Akt catalyses the phosphorylation of Ser136.[61] In a previous study we showed that aPKC-ζ is required for the MEK-mediated activation of ERK in HC11 cells.[31a] These findings would explain the function of aPKC-ζ in Bad phosphorylation at Ser112, which had been shown to be sufficient for heterodimerisation with 14–3–3-protein, resulting in a release of Bcl-2/xL and a subsequent increase in the Bcl-2/Bax ratio.[62] However, blockade of ERK-activation by the MEK inhibitor PD98059 did not affect cis-DDP-induced phosphorylation of Ser112 of Bad (Schwaiger, unpublished), pointing to alternative mechanisms for Bad phosphorylation. Interestingly, however, activation of aPKC-ζ results in a depression of Akt/PKB activity,[63] an observation confirmed in our laboratory. Thus, aPKC-ζ may be able to phosphorylate Bad at the critical serine residues 112 and 136 independently of ERK or Akt, representing a mechanism for a more selective control of cell survival.

Perspectives

The observations described above are part of a tentative model in which atypical PKC isotypes play a fundamental role in balancing cell proliferation and programmed cell death, cellular architecture and cell polarity. However, the criteria that determine whether a particular kinase qualifies as a putative drug target are complex and depend on delicate structural and functional distinctions. Although 3D structure-based design is a powerful technique, no crystal structures for atypical PKC isotypes are available so far. Therefore, most of the studies are based on kinase homology models (e.g., PKA), which can only approximate the actual target structure. In this context, 3D knowledge of structural specificities within critical kinase domains should be helpful. Atypical PKC-ι differs from classical and novel PKCs in the catalytic domain, representing a new aspect of an ATP-binding mechanism and probably catalysis, which should be useful for the development of isotype-specific pharmacophores. Moreover, atypical PKCs possess a so-called OPCA motif, possessing novel regulatory domain characteristics that can be exploited for pharmaceutical intervention by, for example, small molecules interfering with the MEK5-ZIP-aPKC interaction as a prerequisite for transcriptional activation of cyclin D1. Results pointing to MEK5 as a key signalling molecule in prostate carcinogenesis further strengthen this assumption.

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The strongest in vitro evidence for aPKC being an important antitumour drug target is the finding that inhibition of aPKCs sensitises tumour cells in culture to widely used chemotherapeutic agents. The fact that aPKC-ζ-mediated phosphorylation and inactivation of pro-apoptotic Bad at the critical serine residues 112 and 136 occurs independently of ERK/Akt offers an additional survival balancing model.

From these findings, we have hypothesised that interruption of the pro-survival function of aPKC-ι mediated through the ATM/c-Abl/aPKC axis is a precondition for a complete reversal of drug resistance to DNA-damaging agents and, vice versa, over-expression of constitutively active aPKC-ι should protect tumour cells against drug-induced cell death. This was indeed the case for cis-DDP, taxol and camptothecin, which points to a fundamental role in survival control by direct activation of the aPKC survival kinases.

However, in view of the fact that Bcr-Abl regulates aPKC-ι transcription through an Elk1 site in the aPKC-ι promoter,[64] other scenarios of Bcr-Abl-mediated chemoresistance are conceivable. Further studies on the biological role of atypical PKC isotypes in various tumour cell systems are needed. In order to elucidate the biological function of the kinase family members, gene expression profiling experiments employing Affymetix technology are currently underway. Knock-out mouse models of aPKC, together with putative adaptor/regulator proteins, should further clarify our picture of aPKC as a target in carcinogenesis.

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Keywords: antitumor agents · drug design · enzyme models · kinases · OPCA

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