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Protein kinase Cι: human oncogene, prognostic marker and therapeutic target

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Abstract

The Protein kinase C (PKC) family of serine/threonine kinases has been the subject of intensive study in the field of cancer since their initial discovery as major cellular receptors for the tumor promoting phorbol esters nearly thirty years ago. However, despite these efforts, the search for a direct genetic link between members of the PKC family and human cancer has yielded only circumstantial evidence that any PKC isozyme is a true cancer gene. This situation changed in the past year with the discovery that atypical protein kinase C iota (PKCι) is a bonafide human oncogene. PKCι is required for the transformed growth of human cancer cells and the PKCι gene is the target of tumor-specific gene amplification in multiple forms of human cancer. PKCι participates in multiple aspects of the transformed phenotype of human cancer cells including transformed growth, invasion and survival. Herein, we review pertinent aspects of atypical PKC structure, function and regulation that relate to the role of these enzymes in oncogenesis. We discuss the evidence that PKCι is a human oncogene, review mechanisms controlling PKCι expression in human cancers, and describe the molecular details of PKCι-mediated oncogenic signaling. We conclude with a discussion of how oncogenic PKCι signaling has been successfully targeted to identify a novel, mechanism-based therapeutic drug currently entering clinical trials for treatment of human lung cancer. Throughout, we identify key unanswered questions and exciting future avenues of investigation regarding this important oncogenic molecule.

Keywords

Atypical protein kinase C; Par6; Phox/Bem1 domain; cancer signaling; cell polarity; hyperproliferation; invasion and metastasis; mechanism-based therapeutics; aurothiomalate

1. Introduction

Protein kinase C (PKC) was discovered thirty years ago as a novel serine/threonine kinase activity generated by proteolytic cleavage (1). The holoenzyme was subsequently found to be physiologically activated by calcium, membrane lipid (particularly phosphatidylserine), and the lipid metabolite diacylglycerol (2). The fact that PKC activity is regulated by lipid-derived second messengers generated by receptor-mediated events places PKC at a critical nexus in many signal transduction pathways that generate the response of cells to their environment (3,4). PKC represents a family of structurally related, protein kinases that play key regulatory

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roles in a large variety of cellular functions, including cellular proliferation, cell cycle control, differentiation, polarity and survival (5,6). The discovery that PKCs are major cellular receptors for the tumor-promoting phorbol esters (2,7) strongly suggested a functional link between PKC and cancer (8) and distinct changes in the pattern of expression and activity of PKC isozymes have been linked to cancer (9). Multiple PKC isozymes participate in various aspects of the transformed phenotype including hyperproliferation, migration, invasion, metastasis, angiogenesis and resistance to apoptosis (9). Altered PKC activity, localization and/or expression has been observed in virtually all tumor types examined and classification of PKC isozyme-specific functions in transformed cells is emerging (9). Several PKC isozymes have been identified as viable therapeutic targets (10,11) and a number of isozyme-selective PKC inhibitors have been developed and are entering clinical use. Recently, the atypical PKC isozyme PKCι was the first PKC isozyme to be identified as a human oncogene. This review will focus on the role of the two atypical PKC isozymes, PKCζ and PKCι, in human cancer. Particular emphasis will be placed on PKCι-mediated oncogenic signaling mechanisms and the discovery of a mechanism-based therapeutic agent that targets oncogenic PKCι.

2. Atypical PKCs: structurally and functionally distinct PKCs involved in cellular proliferation, survival and polarity

The atypical protein kinase C (aPKC) isozymes PKCι and PKCζ define a sub-class of PKCs that are structurally and functionally distinct from other PKCs (12). The aPKCs differ from other PKCs in that their catalytic activity is not dependent upon diacylglycerol, calcium or phosphatidylserine (7,13). This functional divergence is due to a unique N-terminal regulatory domain on aPKCs that lacks calcium, phospholipid and diacylglycerol binding motifs (7). aPKC activity can be regulated by 3-phosphoinositides (14), phosphorylation by the phosphoinositide-dependent kinase, PDK1 (15-17), and through specific protein-protein interactions (7,12,18-21). aPKCs have been implicated in establishment of cell polarity, cell proliferation and cell survival (12,22-24). aPKCs are thought to be coupled to these diverse cellular functions through direct protein-protein interactions between aPKC and effector molecules mediated through a Phox Bem 1 (PB1) domain (25) within the regulatory region of aPKCs. The PB1 domain is a structurally-conserved, modular protein-protein interaction domain found on a family of signaling molecules (reviewed in (26)). PB1 domains mediate protein-protein interactions through interaction codes that direct specific homo- and hetero-interactions between PB1 domain-containing proteins (27). Perhaps the best characterized PB1 domain interaction involving aPKCs is the polarity complex consisting of aPKC, the polarity protein par6 and a small molecular weight GTPase, either Rac1 or cdc42 (20,21).

PKCζ and PKCι exhibit 72% sequence homology at the amino acid level (28). This striking homology, coupled with the fact that many commercial immunological reagents do not distinguish these closely related isoforms, has made it difficult to biochemically distinguish between PKCζ and PKCι, and as a result many studies on aPKCs are ambiguous with respect to the specific isozyme being evaluated. This is a critical issue since there is definitive evidence, using more recently developed aPKC isozyme-specific immunological reagents and genetic disruption, that PKCζ and PKCι are not functionally redundant. First, expression profiling demonstrates that PKCζ and PKCι exhibit distinct patterns of expression in various tissues and cell types; PKCι is ubiquitously expressed whereas PKCζ exhibits a much more restricted pattern of expression (29). Second, genetic disruption of the PKCζ and PKCι/λ genes has very different effects on embryonic development in the mouse. Knock out of PKCι/λ is embryonic lethal (30), whereas knock out of PKCζ results in viable mice that develop essentially normally, exhibiting only subtle immunological deficiencies (31,32). Third, PKCζ and PKCι/λ preferentially couple to distinct downstream signaling pathways. Using mouse embryo fibroblasts generated from either PKCζ or PKCι knockout mice, it has been shown that PKCζ couples more efficiently to the NF-κB pathway, a well-characterized downstream

effector pathway of aPKCs, than does PKCι/λ (31). Thus, PKCζ-deficient fibroblasts exhibit defects in NF-κB signaling, whereas PKCι/λ-deficient fibroblasts do not. Consistent with its role in NF-κB signaling, PKCζ knockout mice exhibit impaired NF-κB and IL-4 signaling that may account for the immunological deficits seen in these mice (32-34). It is unclear whether the critical role of PKCι/λ in mouse development relates to its role in establishing cellular polarity or to some other critical PKCι/λ-specific embryonic functions.

3. Atypical PKCs in human cancer

Not surprisingly, aPKCs have been implicated in the malignant behavior of transformed human cells. However, just as PKCζ and PKCι play distinct role in normal physiology and embryonic development, they also play distinct, non-overlapping roles in transformed cells. In human myeloid U937 leukemia cells, forced over-expression of PKCζ leads to a longer doubling time, lower saturation density at confluence, and increased adherence to plastic (35,36). PKCζ also induces changes in cellular morphology, surface antigen expression, and lysosomal enzyme activities indicative of a more differentiated phenotype in these cells (36) as well as an enhanced apoptotic response to phorbol ester treatment (35). PKCζ also stimulates motility and maturation of human CD34+ hematopoietic stem and progenitor cells (37). Forced expression of PKCζ also causes decreased anchorage-independent transformed growth, increased differentiation and enhanced apoptosis in CaCo2 human colon cancer cells (38). Interestingly, PKCζ expression is decreased in azoxymethane (AOM)-induced colon tumors in rats, and expression of a dominant negative, kinase-deficient PKCζ (kdPKCζ) mutant inhibits soft agar growth of CaCo2 cells (38), suggesting that loss of PKCζ expression may promote transformed growth and colon tumor formation. Over-expression of PKCζ also inhibits growth of human MDA-MB-468 breast cancer cells (39). In contrast, PKCζ reportedly stimulates directed motility of human MDA-MB-231 breast cancer cells (40) and pancreatic cancer cells (41). However, these results must be interpreted with caution as the assignment of these cellular effects to PKCζ is based solely on the use of pharmacologic small molecule and/or pseudo-substrate peptide inhibitors that are not specific for PKCζ. Therefore, these cellular effects may be attributable to inhibition of other PKC isozymes such as PKCι and possibly other kinase activities.

4. Protein Kinase Cι: A critical enzyme in Bcr-Abl-and Ras-mediated transformation

In contrast to PKCζ, PKCι has been directly implicated in the promotion of carcinogenesis both in vitro and in vivo (22,23,42-44). PKCι, but not PKCζ, is highly expressed in human K562 chronic myelogenous leukemia (CML) cells and genetic disruption of PKCι either by expression of a PKCι-specific antisense construct or a kinase-deficient, dominant negative PKCι mutant (kdPKCι) blocks transformation by Bcr-Abl, and makes these cells extremely sensitive to chemotherapy-induced apoptosis (22). PKCι also promotes colon carcinogenesis in vitro and in vivo (42). PKCι expression is elevated in AOM-induced colon tumors in mice, as well as in human colon carcinomas. Transgenic mice that express constitutively active PKCι (caPKCι) in the colonic epithelium develop more preneoplastic lesions in the colon, aberrant crypt foci (ACF) than non-transgenic littermates after exposure to AOM. In contrast, transgenic mice expressing kdPKCι in the colon develop far fewer ACF (42). ACF are likely precursors to colon cancer in both mice and humans (45,46) and contain many of the same genetic and biochemical alterations found in colon tumors, including increased expression of PKCβII (47) and activating K-Ras mutations [43]. Both the number and multiplicity (number of crypts/focus) of ACF are highly predictive of subsequent colon tumor formation in rodents (48) and ACF correlate with increased risk of colon cancer in humans (46). As expected, transgenic caPKCι mice exhibit a three-fold higher incidence of colon tumors than non-transgenic control mice (42). Interestingly, transgenic caPKCι mice develop mostly malignant

carcinomas whereas non-transgenic mice develop mainly benign tubular adenomas (42). Thus, elevated PKCι activity in the colonic epithelium promotes formation of preneoplastic ACF and subsequent colon tumors, and promotes colon tumor progression from benign adenoma to malignant carcinoma in vivo (42).

PKCι has also been directly linked to oncogenic Ras signaling (49-52). Ras can bind and activate aPKCs (53,54) and PKCι has been implicated in oncogenic Ras signaling in fibroblasts (49-51,55). PKCι also plays a critical role in oncogenic Ras-mediated transformation in the intestinal epithelium (42). Expression of wild type PKCι significantly enhances, and kdPKCι blocks, both invasion and soft agar growth of Ras-transformed rat intestinal epithelial (RIE/Ras) cells (42). PKCι is also required for Ras-mediated colon carcinogenesis in vivo (42). K-Ras^{LA} mice containing a latent oncogenic K-Ras allele (G12D) that is activated by spontaneous recombination in vivo develop K-Ras-dependent lung carcinomas and ACF in the colonic epithelium (56). When K-Ras^{LA} mice are crossed to transgenic kdPKCι mice, bitransgenic K-Ras^{LA}/kdPKCι mice develop significantly fewer ACF in the colon than K-Ras^{LA} mice, consistent with the results in RIE/Ras cells in vitro (42).

5. PKCι is required for transformed growth and tumorigenicity of human non-small cell lung cancer cells

Somatic mutations in Ras are among the most frequent oncogenic changes observed in human epithelial tumors, and are estimated to be present in 30% of all human cancers, including the majority of non-small cell lung cancers (NSCLC). PKCι is highly expressed in NSCLC cells compared to non-transformed lung epithelial cells whereas PKCζ was undetectable in both non-transformed lung epithelial and NSCLC cells (57). Expression of kdPKCι in human A549 lung adenocarcinoma (LAC) cells, which harbor an oncogenic K-ras allele, blocks anchorage-independent growth in soft agar (22,42). Interestingly, no significant change in growth rate, saturation density or survival is observed in A549/kdPKCι cells compared to A549 cells expressing a control plasmid (A549/pBabe cells) when grown in adherent culture, indicating that PKCι is dispensable for adherent cell growth and survival, but is required for anchorage-independent transformed growth (57). PKCι is also required for transformed growth of other NSCLC cell lines that do not harbor K-ras mutations, indicating that the requirement for PKCι in transformed growth is not restricted to cells harboring oncogenic K-ras mutation (57,58). Expression of kdPKCι also inhibits the tumorigenicity of NSCLC cells in vivo indicating that the effects of PKCι are not restricted to tumor cell behavior in vitro (57). kdPKCι inhibits tumor cell proliferation in vivo without apparent effects on tumor cell survival, apoptotic rate or tumor vascularity (57). Thus, the primary function of PKCι in NSCLC tumor is to drive transformed growth, consistent with results in colonic epithelial cells (42). PKCι also is required for transformed growth of ovarian cancer cells in soft agar but not their growth in adherent culture (59). Therefore, PKCι appears to participate in signaling pathways specifically required for transformed cell growth but not for adherent cell growth.

PKCι also functions in NSCLC cell survival, resistance to chemotherapy and invasion in vitro (60). Inhibition of PKCι expression causes A549 cells to undergo apoptosis in response to the smoke carcinogen Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) as well as the chemotherapeutic agents taxol and cis-platin (60). These results are consistent with those observed in CML cells, in which PKCι inhibition causes increased sensitivity to taxol-mediated apoptosis (22,23). Over-expression of PKCι enhances, and inhibition of PKCι expression inhibits, migration and invasion of NSCLC cells in response to nicotine (60), similar to the effects observed in ras-transformed intestinal epithelial cells (42). Thus, PKCι plays a critical role in the control of anchorage-independent growth, resistance to chemotherapeutic agent-and carcinogen-induced apoptosis, cellular motility and invasion in multiple human cancer cell types.

6. PKCι is a prognostic marker and oncogene in non-small cell lung and ovarian cancer

aPKC expression has been extensively analyzed in primary human NSCLC and ovarian tumors. PKCι mRNA and protein is over-expressed in the vast majority of primary NSCLC tumors (~70%) whereas PKCζ mRNA and protein is extremely low or undetectable in both normal and cancerous lung tissues (58). Immunohistochemistry reveals that PKCι overexpression is predominantly confined to lung tumor cells, with little or no expression in tumor-associated stroma (58). PKCι expression is highly prognostic of poor clinical outcome; NSCLC patients with elevated tumor PKCι are 2.6 times more likely to die from cancer than patients without elevated PKCι (58). PKCι is as good of a prognostic indicator as tumor stage, which is currently the best prognostic indicator in NSCLC (61). Importantly however, PKCι expression does not correlate with tumor stage in NSCLC, since PKCι levels are comparable in tumors from NSCLC patients with early and late stage disease (58). Strikingly, patients with early stage lung cancer and high PKCι are almost 11 times more likely to die from their disease than those with low PKCι (58). Therefore, PKCι expression profiling can identify patients with early stage lung cancer at elevated risk of relapse (58). Since ~40-50% of patients diagnosed with early stage lung cancer will eventually relapse, PKCι expression profiling can be used to identify high risk patients who would be candidates for more aggressive clinical management, perhaps with PKCι-targeted therapy.

Elevated PKCι is also frequently observed in ovarian cancer patients (59,62,63). Just as in NSCLC, PKCι expression correlates with poor clinical outcome of ovarian cancer patients, however in ovarian cancer, PKCι expression correlates with tumor stage, suggesting that PKCι may contribute to ovarian tumor progression and aggressiveness (59,62,63). High PKCι correlates with increased cyclin E expression and together these two markers predict poor clinical outcome, increased proliferation and defects in polarity in nonserous epithelial ovarian cancers (62). It is unclear at present whether there is a direct functional link between PKC and cyclin E expression in these tumors. It is interesting that PKCι correlates with stage in ovarian cancer but not lung cancer. This observation suggests that PKCι over-expression is a later event in ovarian carcinogenesis than in lung carcinogenesis. Thus, PKCι may play a distinct role in the development and progression of these two tumor types. Alternatively, this apparent discrepancy may relate to differences in the staging and/or natural progression of these diseases. The relatively high relapse and metastasis rates observed in lung cancer patients diagnosed with early stage disease suggest that a significant number of these patients may be inaccurately staged using current methods. In this regard, PKCι expression profiling may be useful not only as a prognostic tool in NSCLC, but also may assist in the development of more accurate staging procedures for this disease.

The frequent over-expression of PKCι in lung and ovarian tumors prompted an investigation into potential molecular mechanisms that could account for elevated PKCι expression in these tumors. In 36% of the NSCLC tumors examined, the PKCι gene, which resides on chromosome 3q26, is amplified in a tumor-specific fashion (58). PKCι gene amplification drives PKCι mRNA and protein expression and correlates with poor outcome in NSCLC tumors (58). Interestingly, PKCι gene amplification was frequently found in lung squamous cell carcinonoma (SCC) (~70%) but rarely in lung adenomcarcinoma (LAC) (58), consistent with the distribution of chromosome 3q26 amplification in NSCLC which is also confined to SCC (64,65). PKCι is required for the transformed growth of SCC cells harboring PKCι gene amplification, indicating that PKCι is an important target of the chromosome 3q26 amplicon (58). Similar tumor-specific PKCι gene amplification has been observed in ovarian cancers, particularly those of the serous sub-type (~70%) (59,62). PKCι expression and PKCι gene copy number correlates with chromosome 3q26 gains in these tumors (59,62) indicating that, as in the lung, PKCι is a relevant gene target for tumor-specific chromosome 3q26 amplification.

Since chromosome 3q26 amplification is one of the most common chromosomal changes in human cancers, and is frequently observed in SCC of the head and neck (66), esophagus (67, 68) and cervix (69), it is likely that PKCι expression and gene copy number is of prognostic significance in these tumors as well. It remains to be determined whether PKCι also plays a critical promotive role in these human tumor types, as it does in lung and ovarian cancers.

PKCι gene amplification is not the sole mechanism by which PKCι expression is elevated in human tumors. PKCι expression is elevated to the same degree, and just as frequently, in lung SCC and LAC despite the fact that PKCι gene amplification is largely confined to SCC tumors (58). Furthermore, PKCι is frequently over-expressed in other tumor types, including colon cancers (42), pancreatic cancers (70), and CML (71) that do not harbor frequent chromosome 3q26 amplification. Though little is known about the transcriptional regulation of PKCι in either normal or transformed cells, we recently demonstrated that Bcr-Abl transcriptionally activates PKCι through a specific Elk1 element within the proximal PKCι promoter in CML cells (71). Whether this transcriptional mechanism plays a role in the control of PKCι expression in other tumor types remains an important area for future investigation. Other potential mechanisms for oncogenic activation of PKCι, such as post-transcriptional regulation, post-translational modifications and/or somatic mutation, have not been exhaustively analyzed and warrant further investigation. In this regard, we have conducted sequence analysis of all 18 exons of the PKCι gene in 20 LAC cases and 20 SCC cases that do not harbor PKCι gene amplification and have failed to detect any mutations, suggesting that somatic mutation of PKCι does not occur or is extremely rare in NSCLC (Regala and Fields, unpublished observation). In summary, PKCι is the first PKC isozyme shown to be a bonafide human oncogene by virtue of the fact that it is activated in human NSCLC and ovarian tumors via a tumor-specific genetic alteration (gene amplification) and it is required for the transformed phenotype of these cells.

7. Oncogenic PKCι signaling mechanisms

It is perhaps not surprising that PKCι functions in multiple signaling pathways involved in different aspects of the transformed phenotype (Figure 1). Major oncogenic signaling axes in which PKCι appears to function will be discussed below.

7.1 PKCι and cell survival signaling

PKCι promotes survival in multiple human tumor cell types, though the signaling mechanisms involved appear to differ in different tumor types. PKCι confers resistance of CML cells to chemotherapy-induced apoptosis through Bcr-Abl-mediated, PKCι-dependent activation of NF-κB (44). In CML cells, PKCι activates NF-κB transcriptional activity by at least two distinct mechanisms. PKCι activates the canonical NF-κB pathway to induce IκB degradation and nuclear translocation and activation of NF-κB (44). PKCι also trans-activates nuclear NF-κB DNA binding and transcriptional activity (44). PKCι (and PKCζ) can directly activate IκK activity in HEK293 cells to induce the canonical NF-κB signaling cascade (72). In fibroblasts, aPKC is activated for this function through engagement of the TNF-R1 (72). aPKC-mediated activation of NF-κB in fibroblasts is dependent upon the scaffolding protein p62, a PB1 domain binding partner of aPKC, which serves to couple aPKC to NF-κB signaling (72). Since HEK 293 cells express both PKCι and PKCζ the relative role of these two aPKCs in NF-κB signaling can not be distinguished (72). However, given the fact that PKCζ but not PKCι is coupled to NF-κB in mouse embryo fibroblasts, it is possible that PKCζ is primarily responsible for NF-κB survival signaling in HEK 293 cells. It remains to be determined whether PKCι couples to NF-κB in human epithelial tumor cells that express predominantly or only PKCι, or whether this effect is specific to Bcr-Abl-transformed CML cells. The role of the p62 scaffolding protein in Iinking PKCι to NF-κB in human tumor cells also remains to be determined.

PKCι also stimulates survival in human NSCLC and glioblastoma cells. In NSCLC cells, carcinogen-induced Src activity activates PKCι, leading to increased tumor cell survival (60). Src can directly bind and induce tyrosine phosphorylation of aPKC leading to its activation (73). In A549 cells, Src-activated PKCι appears to directly phosphorylate the pro-apoptotic protein Bad at sites that prevent its interaction with Bcl-XL (60). In this model system, phosphorylated Bad no longer binds to and suppresses Bcl-XL activity, leading to enhanced survival and/or chemoresistance to smoke carcinogen (60). In human glioblastoma cells, RNAi-mediated knock down of PKCι leads to increased sensitivity to cis-platin-induced apoptosis (74). This effect appears to be mediated through loss of PKCι-dependent attenuation of p38 MAP kinase signaling. Thus, PKCι couples to at least three distinct survival pathways in human cancer cells. It should be noted that whereas PKCι activates the BAD-mediated survival pathway in A549 human NSCLC cells (60), it does not appear to couple to the NF-κB survival pathway in these cells (57). It remains to be determined whether these survival pathways can function together in some tumor cells and if so, to what extent each contributes to tumor cell survival.

7.2 PKCι and oncogenic Ras signaling

Oncogenic Ras can activate atypical PKCs (53,54) and PKCι is required for oncogenic Ras-mediated signaling (49-51). Bcr-Abl transcriptionally regulates PKCι expression through Ras/Mek-dependent activation of an Elk1 element within the proximal PKCι promoter (71). PKCι is in turn necessary for Bcr-Abl-mediated chemoresistance in CML cells (71) mediated through the NF-κB survival pathway (44). PKCι activity is also required for Ras-mediated transformation, invasion and anchorage independent growth of intestinal epithelial cells, and is critical for Ras-and carcinogen-mediated colon carcinogenesis in vivo (42,43). Similarly, PKCι signaling is required for the transformed growth of multiple human NSCLC cell lines, including those that harbor K-ras mutation (57).

PKCι also plays an essential role in the pro-carcinogenic effect of another PKC isozyme, PKCβII. PKCβII expression is elevated very early in colon tumors and PKCβII expression is required for AOM-induced colon carcinogenesis (47,75,76). Expression of PKCβII in the colon of transgenic mice induces hyperproliferation and increased susceptibility to colon cancer (75). Expression of PKCβII in RIE cells induces an invasive phenotype that is dependent upon PKCι (43). PKCβII induces invasion through activation of K-Ras and the Ras effector, Rac1, in RIE/PKCβII cells (43). PKCβII-mediated invasion is blocked by the Mek inhibitor, U0126, and by expression of either dominant negative Rac1 or kdPKCι. Expression of constitutively active Rac1 induces Mek activity and invasion, indicating that PKCβII induces invasion through a novel PKCβII→Ras→PKCι/Rac1→Mek signaling pathway (43). PKCι has also been implicated in the invasive phenotype exhibited by human NSCLC cells (77). In NSCLC cells, PKCι can directly phosphorylate μ- and m-calpains which are associated with increased wound healing, migration and invasion (77). It remains to be determined whether PKCι mediates cell migration through common or distinct signaling pathways in different tumor cell types. In this regard, Rac1 is a critical effector of PKCι-mediated transformed growth and invasion in NSCLC cells (57,58,78). Expression of kdPKCι leads to inhibition of Rac1 activity and loss of anchorage-independent growth and tumorigenicity of NSCLC cells in nude mice. Expression of a constitutively active Rac1 allele, RacV12 restores soft agar growth and tumorigenicity in cells expressing kdPKCι (57). Further studies demonstrated that PKCι activates a Rac1→p21-activated kinase (Pak)→Mek→Erk signaling axis that is required for transformed growth and invasion (57). Taken together, both Rac1 and NF-κB have been shown to be downstream effectors of oncogenic PKCι signaling, coupling PKCι to transformed growth, cellular invasion and survival signaling.

8. The Phox/Bem1 domain in oncogenic PKCι signaling

Both Rac1 and NF-κB are important effectors of oncogenic PKCι signaling. aPKCs are thought to be coupled to these signaling molecules via protein-protein interactions involving the PB1 domain of aPKC (25). At least three PB1 domain-containing proteins have been identified that bind to aPKCs via PB1-PB1 domain interactions; p62 (18,79), par6 (21,80-82), and Mek5 (79,83). p62 is a scaffold protein that links aPKC to NF-κB downstream of extracellular signals such as tumor necrosis factorα, interleukin-1 and the nerve growth factor (72,84,85). Mek5 is a member of the mitogen-activated kinase family implicated in cellular transformation and cancer cell survival at least in part through activation of NF-κB (86-89). Mek5 has also been shown to bind aPKCs and participate in mitogenic EGF signaling in HEK 293 cells (83). However, it remains to be determined whether Mek5 and p62 participate in oncogenic PKCι signaling in human cancers.

The scaffold protein par6 binds aPKC and links it to cell polarity by forming a core complex consisting of aPKC, par6, and either of the small molecular weight GTPases, Rac1 or Cdc42 (20,21,25,80-82,90). The PB1 domain of par6 heterodimerizes with the PB1 domain of aPKC (91) while a CRIB motif on par6 binds the GTP-bound forms of Rac1 or Cdc42 (21,80,81). The aPKC-par6 polarity complex is required for asymmetric cell division (92), directed cell migration (93), epithelial tight junction formation (94), cell adhesion, cytoskeletal reorganization and scaffolding of signaling complexes (95). In polarized epithelial cells the apical membrane is segregated from the basolateral membranes by tight junctions. The aPKC-Par6 polarity complex plays a critical role in establishment of this apical-basolateral polarity. Cellular polarity is critical for epithelial cell function and disruption of cell polarity is a key feature of the transformed phenotype. Loss of apical-basal cell polarity is required for epithelial-mesenchymal transition (EMT), a critical step in the acquisition of cellular motility and invasiveness observed in transformed tumor cells (96). Loss of polarity also disrupts tight junctions normally found in polarized cells which serve to segregate many growth factors from their receptors preventing autocrine activation (97). Interestingly, several members of the polarity complex, including PKCι, Rac1 and cdc42 have well-documented oncogenic potential. Rac1 is a critical effector of PKCι-mediated transformation (57) and expression of the PB1 domain of PKCι uncouples PKCι from Rac1, blocks PKCι-mediated Rac1 activity and inhibits transformed growth, indicating that the PB1 domain of PKCι is required for oncogenic PKCι signaling (57). At present, it is unclear which, if any, of the previously identified PB1-PB1 domain interactions involving PKCι are directly involved in its transforming properties. However, the fact that Rac1 is required for PKCι-dependent transformation directly implicates the PKCι-par6-Rac1 polarity complex in oncogenic signaling.

9. Targeting oncogenic PKCι signaling for treatment of human cancer

The fact that PKCι is an oncogene required for the transformed growth and tumorigenicity of human tumor cells suggest that it is an attractive target for development of novel mechanism-based therapeutics for NSCLC and other human cancers. Since the PKCι-par6 polarity complex has been implicated in PKCι-mediated oncogenic signaling we developed a novel fluorescence resonance energy transfer (FRET)-based assay to screen for compounds that can disrupt the PB1-PB1 domain interaction between PKCι and par6 (78) (Figure 2A). A high throughput screen of a commercial small molecule library consisting of ~1,000 compounds used in clinical practice identified the gold compound aurothioglucose (ATG) as a potent inhibitor of the PB1 domain-mediated interaction between PKCι and par6 (78). ATG (Figure 2B), and the related compound aurothiomalate (ATM) (Figure 2C), exhibit dose dependent inhibition of PKCι-par6 binding with an apparent IC_{50} in the low micromolar range (78). Furthermore, both ATG and ATM block PKCι-mediated signaling to Rac1 and transformed growth of NSCLC cells in vitro and tumorigenicity in vivo (78).

10. Aurothiomalate (ATM) inhibits transformation by targeting the PB1 domain of PKCι

ATG and ATM have been used for many years in the treatment of rheumatoid arthritis but their use has become limited recently with the development of more effective compounds (98). Despite extensive use in clinical practice, the precise mechanism of action of gold compounds in rheumatoid arthritis is unknown. Thio-gold compounds such as ATG and ATM can form gold-cysteine adducts on target cellular proteins. ATM can inhibit the activity of thioredoxin reductase through formation of a gold adduct with a critical cysteine residue within the active site of the enyzyme and this mechanism has been suggested to play a role in the anti-oxidant effects of ATM (99). These compounds also exhibit potent anti-inflammatory properties thought to be mediated through inhibition of NF-κB signaling (100,101). While the molecular mechanism underlying the ability of these compounds to inhibit NF-κB signaling has not been definitively elucidated, a recent report suggests a similar mechanism involving a critical cysteine residue in IκK (102). However, despite the fact that ATM can modify these target proteins, and thereby inhibit their activity, there is no conclusive evidence that these proteins are the relevant target(s) for the anti-rheumatic effects of ATM in vivo.

Since cysteine residues may be important targets for ATM action, we assessed whether the PB1 domains of PKCι and/or par6 contain cysteine residues that could serve as potential targets for ATM binding. Alignment of the sequences of human PB1 domains reveals a unique cysteine residue in aPKCs, Cys69, located within the conserved OPR, PC and AID (OPCA) motif responsible for binding to par6 and p62 (Figure 3A). Interestingly, Cys69 is located at the binding interface between PKCι and par6 where it interacts with Arg28, a residue within the basic cluster of par6 involved in PKCι binding (27, 79) (Figure 3B). Mutation of Cys69 in PKCι to either isoleucine (C69I) or valine (C69V), the two amino acids most frequently seen at this position in other PB1 domains, has no effect on par6 binding (78). However, the C69I and C69V PKCι mutants exhibit almost complete resistance to the inhibitory effects of ATM on par6 binding in vitro (78) (Figure 3C). Furthermore, expression of the C69I PKCι mutant in NSCLC cells renders them resistant to the inhibitory effects of ATM on transformed growth (78) demonstrating that Cys69 is a critical target for the inhibitory effects of ATM on transformed growth (Figure 3D). Molecular docking of ATM onto the PKCι PB1 domain crystal structure predicts formation of a cysteine-aurothiomalate adduct at Cys69 that protrudes into the binding cleft between PKCι and par6 causing steric hinderance to par6 binding (78) (Figure 4).

The identification of Cys69 as a critical target for ATM action has several important implications for ATM as a therapeutic agent for treatment of cancer. First, it predicts that ATM will selectively inhibit PB1 domain interactions involving PKCι (both with par6 and p62) but not other PB1-PB1 domain interactions. Consistent with this prediction, ATM inhibits both PKCι-par6 and PKCι-p62 binding, but not p62-p62, p62-NBR1 or MEK5-MEKK3 PB1-PB1 domain interactions (78). Taken together, these data demonstrate that a major mechanism by which ATM inhibits PKCι-par6 and PKCι-p62 interactions is through direct binding to Cys69 within the OPCA motif of PKCι. Since ATM can inhibit both PKCι-par6 and PKCι-p62 interactions it should be effective at uncoupling PKCι from both the Rac1 and NF-κB effector pathways. Likewise, ATM should be an effective inhibitor of PKCζ-mediated functions that rely on PB1 domain interactions such as NF-κB activation. These results are interesting in light of the fact that ATM has long been known to inhibit NF-κB signaling (100-102), and provide a plausible unifying molecular mechanism that could account for both the anti-rheumatic and anti-tumor activity of ATM. We are currently conducting a phase I clinical trial of ATM in NSCLC patients.

11. Conclusions

The two aPKC isozymes PKCι and PKCζ play distinct non-overlapping roles in human cancer. Whereas PKCζ inhibits aspects the transformed phenotype, PKCι is promotive of transformed growth, invasion, chemoresistance, and tumor cell survival. PKCι is frequently over-expressed and is a frequent target for tumor-specific genetic alteration by gene amplification in multiple human tumor types, making it the first PKC isozyme to be identified as a bonafide human oncogene. PKCι expression profiling is a useful prognostic marker of poor clinical outcome in several human cancers, and also shows promise as a method to more accurately stage non-small cell lung cancer patients. Oncogenic PKCι signaling is complex, and may exhibit tumor type specificity. The PKCι-par6 polarity complex is directly implicated in oncogenic PKCι signaling and this complex has been successfully targeted in the development of a novel, mechanism-based therapy that is being clinically evaluated for the treatment of non-small cell lung cancer.

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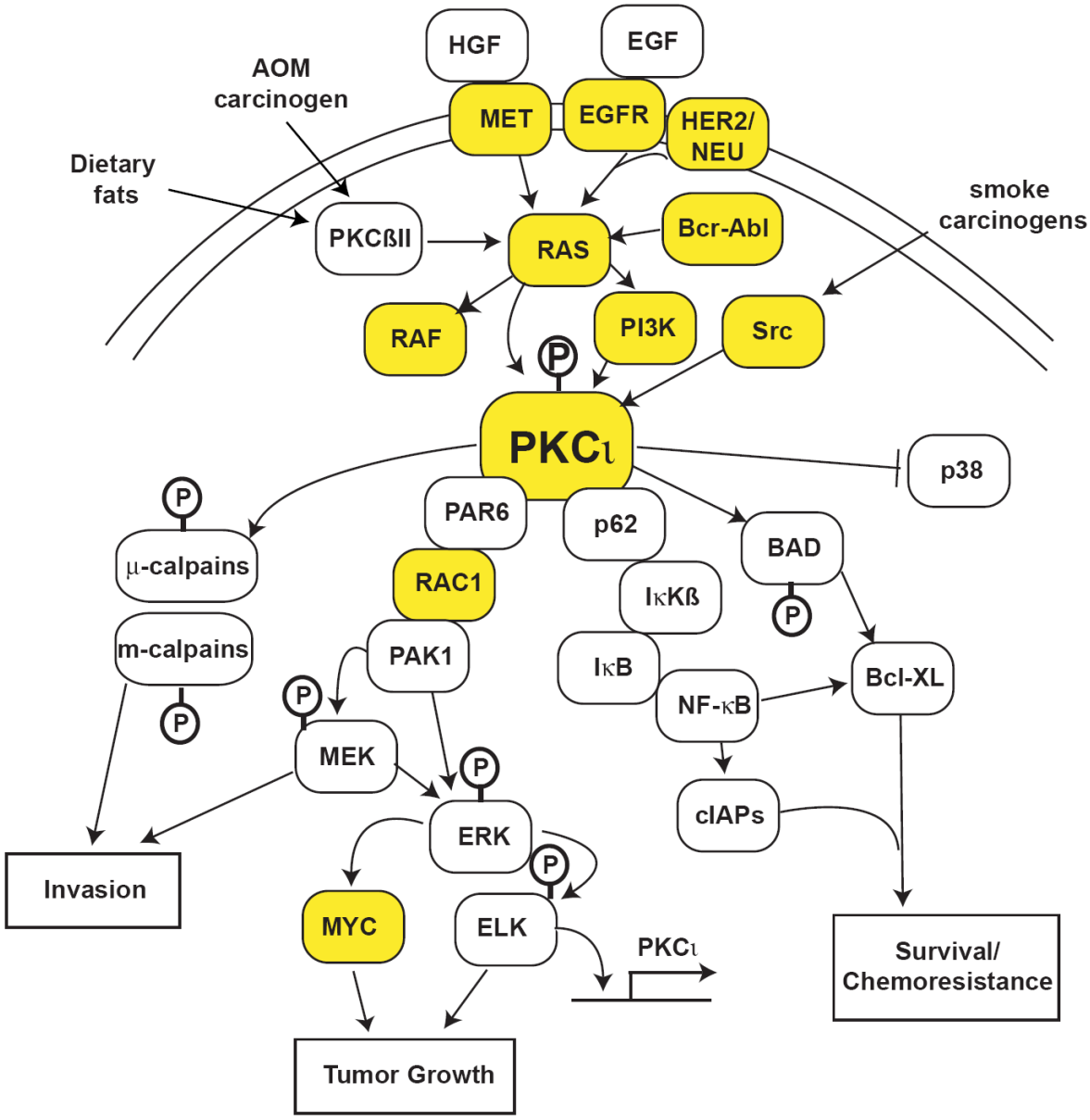

Figure 1. Schematic of major oncogenic PKCι signaling pathways

PKCι resides in several major signaling pathways implicated in human cancer. Many components of these pathway are mutated, often by multiple mechanisms (ie. gene amplification and somatic mutation), in human tumors (indicated by yellow boxes). Arrows indicate flow through signaling pathways; touching boxes indicate directly binding of signaling components. Phosphorylation events are indicated by circled Ps.

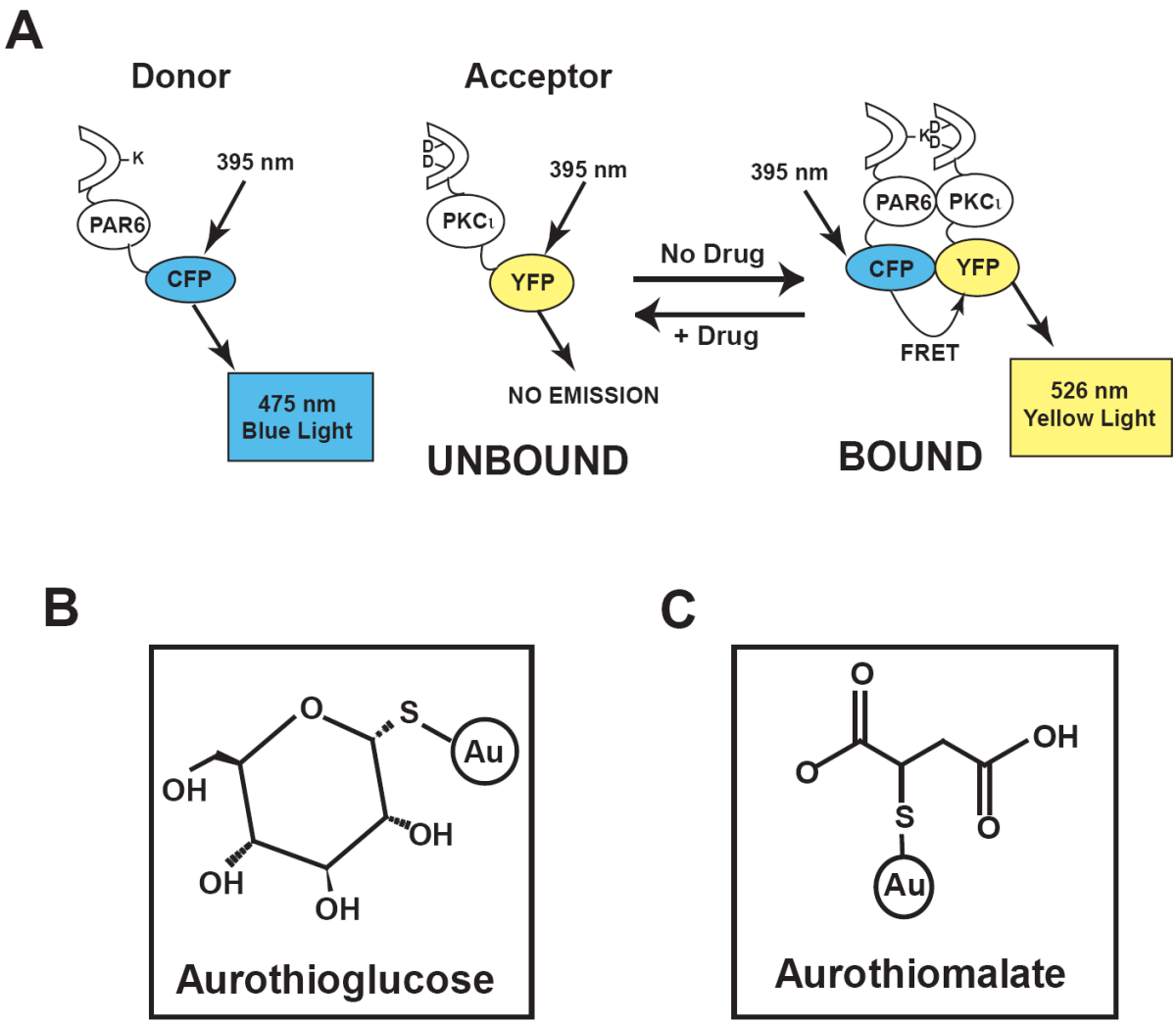

Figure 2. Schematic diagram illustrating the basis of a FRET-based assay to screen for inhibitors of the Par6/PKCι interaction

(**A**) When exposed to 395 nm light, the donor molecule Par6/CFP, emits blue fluorescent light with an emission maximum at 475 nm. The acceptor molecule PKCι/YFP does not fluoresce when exposed to 395 nm light. When Par6/CFP and PKCι/YFP are bound, excitation of the donor with 395 nm light leads not only to emission of blue light at 475 nm, but also to FRET-mediated emission of yellow light at 529 nm. In the presence of a chemical inhibitor such as ATG or ATM, FRET is blocked. Chemical structures of ATG (**B**) and ATM (**C**), respectively.

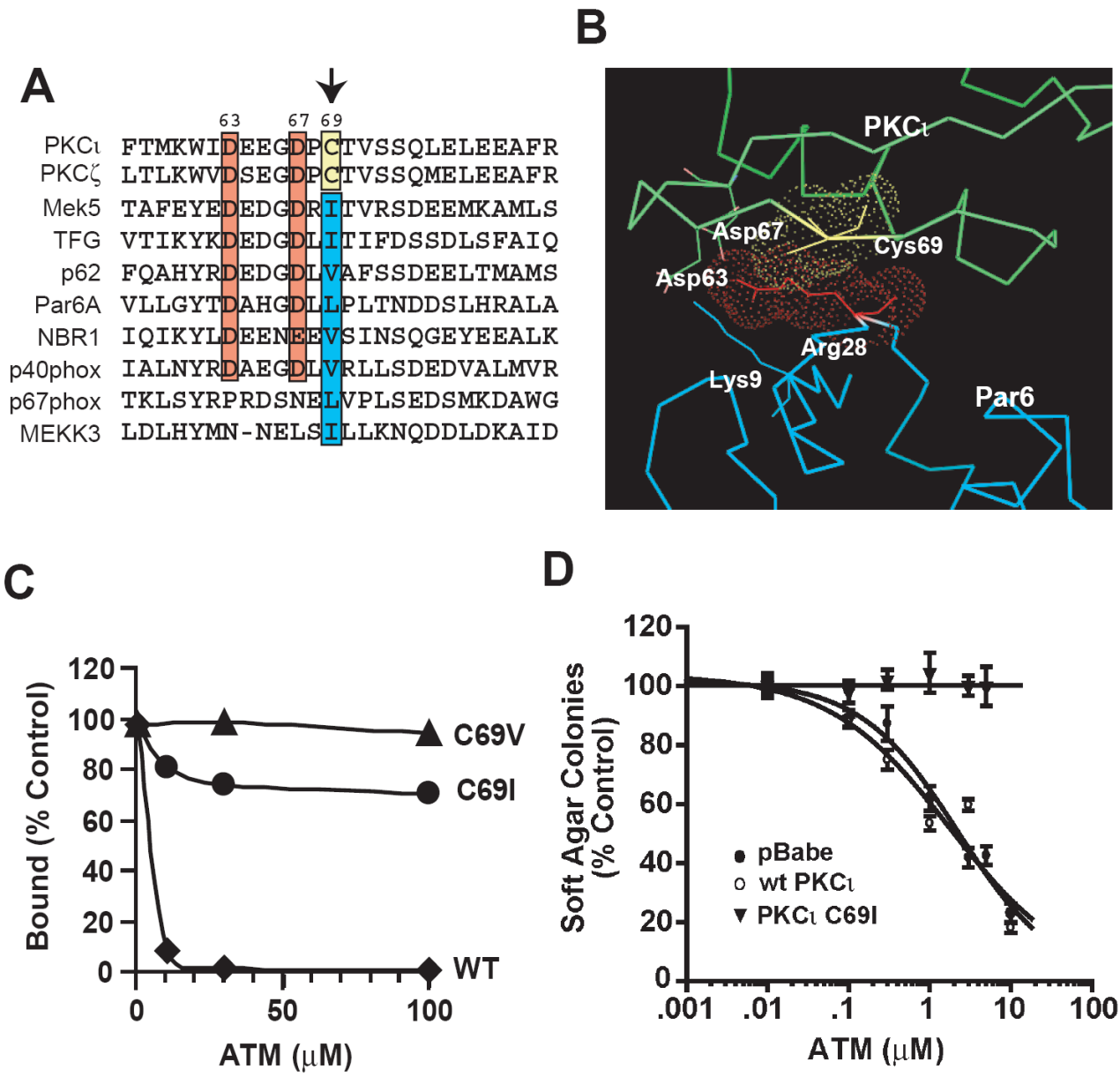

Figure 3. ATM targets Cys 69 on PKCι to inhibit PKCι-par6 binding and PKCι-dependent transformed growth

(**A**) Sequence alignment of the OPCA motifs of 10 human PB1 domain-containing proteins reveals that cysteine 69 (Cys69) is unique to the OPCA motif of PKCι and PKCζ. (**B**) The crystal structure of the PKCι-Par6complex reveals that Cys69 of PKCι resides in the binding interface between PKCι (*green*) and Par6 (*blue*) where it interacts with Arg28 on Par6. (**C**) Mutation of Cys69 on PKCι to isoleucine or valine abolishes the inhibitory effect of ATM on Par6 binding. (**D**) NSCLC cells stably expressing a PKCι C69I mutant are resistant to the inhibitory effects of ATM on transformed growth.

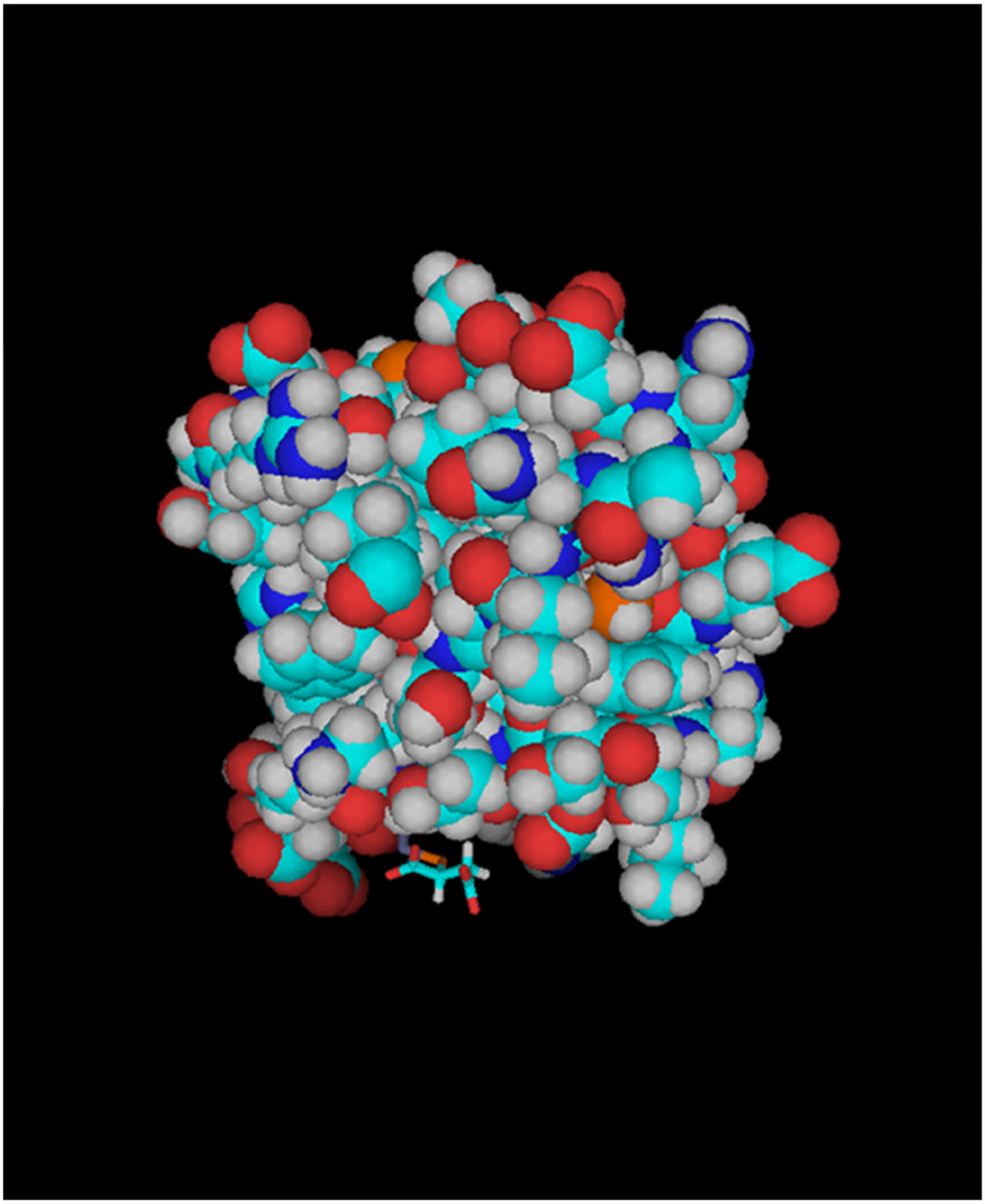

Figure 4. Molecular model of the PKCι-Cys69-ATM adduct based on the crystal structure of the PKCι PB1 domain

The Cys69-ATM adduct protrudes into the binding cleft normally occupied by Par6 in the PKCι-Par6 complex (*bottom of structure*).