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Abstract

Protein kinase C (PKC) is a family of serine/threonine protein kinases that plays a central role in transducing extracellular signals into a variety of intracellular responses ranging from cell proliferation to apoptosis. Nine PKC genes have been identified in the human genome, which encode 10 proteins. Each member of this protein kinase family displays distinct biochemical characteristics and is enriched in different cellular and subcellular locations. Activation of PKC has been implicated in the regulation of cell growth and differentiation. This review summarizes works of the past years in the field of PKC biochemistry that covers regulation and activation mechanism of different PKC isoforms.

Introduction

Protein kinase C (PKC) was identified as a receptor of phorbol esters by Yasutomi Nishizuka and colleagues at the National Institute of Basic Biology in Japan and Peter Blumberg and colleagues at the National Cancer Institute in USA in the early of 1980s (Castagna et al., 1982; Leach et al., 1983). Phorbol esters are natural products which had long been known as cancer-promoting agents but the molecular targets of these natural products remained unknown until these discoveries. These discoveries brought PKC to the forefront of the cancer research field and consecutive cloning of many structurally related PKC isoforms disclosed a family of protein kinases. PKC is conserved in eukaryotes from yeast to human (Kazi et al., 2008; Mellor and Parker, 1998). The PKC family members are involved in the signal transduction events of several critical pathways including cell proliferation, differentiation and apoptosis (Mackay and Twelves, 2007). Despite three decades of extensive investigation since discovery of PKC as a phorbol esters receptor, there is only limited knowledge on isoform-specific PKC substrates and their relationship to these events. Here we discuss progress in the field of PKC biochemistry including structure, activation mechanism and regulation.

PKC family members

PKC represents a family of 10 protein serine/threonine kinases which is encoded by nine

genes. PKC isoforms are members of AGC group kinase which can be broadly subdivided into three subfamilies considering their domain structure and cofactors requirement. Classical or conventional PKC isoforms are regulated by phospholipids, diacylglycerol (DAG) and calcium that include PKCα, PKCβ2 and its transcription variant PKCβ1, and PKCγ. PKCδ, PKCε, PKCη and PKCθ are known as novel PKC isoforms which are regulated by phospholipids and DAG. PKCζ and PKCι are categorized as atypical PKC isoforms which are independent to DAG and calcium, but can be regulated by phospholipids. PKC proteins contain a poorly conserved amino-terminal regulatory domain and a highly conserved carboxy-terminal catalytic domain (Fig. 1).

PKC regulatory domain

The PKC regulatory domain contains several regulatory regions such as pseudosubstrate region, protein kinase C homology 1 (C1) domain, protein kinase C homology 2 (C2) domain and PB1 domain. This domain is the target of lipid second messengers such as DAG and other phorbol esters. Although the 3D structure of the intact regulatory domain of PKC isoforms has not been determined yet, the crystal or solution structure of individual domains of several PKC isoforms has been resolved and the function of each of these domains has been established by extensive biochemical analysis.

Pseudosubstrate region: In the late 1980s House and Kemp first proposed that PKC isoforms contain a pseudosubstrate region when they identified 17 amino acid residues near the N-terminal of regulatory domain as a potent PKC substrate agonist (House and Kemp, 1987). They also found that a single alanine to serine mutation in this region changes this peptide to the potent PKC substrate. A study with an antibody against this sequence put further evidence that the pseudosubstrate region is responsible for maintaining the enzyme in an inactive state (Makowske and Rosen, 1989). A conserved arginine residue in this region was shown to be important for the high affinity binding with the active site (House and Kemp, 1990). Thus the activity of PKC isoforms is regulated by pseudosubstrate region, which blocks the substrate binding site of the enzyme in absence of agonists.

C1 domain: Classical and novel PKC isoforms localize to the membrane in response to increased DAG level resulting in activation of these isoforms. Membrane localization is fabricated by the direct binding of DAG to the C1 domain. The C1 domain is a zinc-containing motif of 50-51 amino acid residues. This domain is also known as cysteine-rich domain. C1 domain is found as a tandem repeat in classical and novel PKC isoforms (Fig. 1) referred to as C1a and C1b domains. Each domain has a conserved motif of cysteine and histidine residues ($HX_{12}CX_2CX_{13}$-

$_{14}CX_2CX_4HX_2CX_7C$). These residues create the coordination sites for two zinc ions. Classical and novel C1 domains exhibit higher affinity for phorbol esters, which are mimics to DAG. Conversely, the atypical C1 domain includes only a C1a domain and does not bind to DAG or phorbol esters. The crystal structure of PKCδ-C1b (Zhang et al., 1995) and the solution structure of PKCγ-C1b (Xu et al., 1997) have been resolved. C1b domain is composed of five β-strands and one α-helix (Xu et al., 1997; Zhang et al., 1995). The top third of the C1 domain is completely devoid of charged groups, while the middle third is mostly composed of positively charged side chains. Two β-strands form a narrow hydrophilic groove and four rings of the phorbol ester insert themselves into the groove. Three phorbol oxygens form five hydrogen bonds with two β-strands of the domain. The ligand covers the hydrophilic binding site of the groove so that the large part of the domain displays a flanking hydrophobic surface. This binding does not produce a significant conformational change within this domain (Zhang et al., 1995). Thus, membrane targeting is achieved by altering the nature of the protein surface rather than by changing protein conformation. One study suggests that C1 domains exhibit differential affinity for DAG and the C1b domain is the major domain for DAG binding (Szallasi et al., 1996). However, exactly how C1 domains respond differentially to the DAG remain unclear. Availability of 3D structure information of C1a domain would be useful to understand this issue.

C2 domain: The C2 domain was discovered as a conserved sequence motif in the classical PKC isoforms that consists of about 120 amino acid residues. The C2 domain is also present in all novel PKC isoforms. Classical C2 domains interact with phospholipid membranes in a Ca^{2+} dependent manner. The conserved aspartate residues are critical for this multiple Ca^{2+} ions binding (Corbalan-Garcia et al., 1999; Medkova and Cho, 1998). In contrast, novel C2 domains lack conserved aspartate residues and interact with acidic phospholipids in a Ca^{2+} independent manner (Corbalan-Garcia et al., 2003). Despite binding to phospholipids, the novel C2 domain has been characterized as phosphotyrosine binding domain that exhibited binding with diverse tyrosine phosphorylated proteins such as CDCP1 and PKD1 (Benes et al., 2005; Doppler and Storz, 2007). High-resolution crystal structures have been determined for the C2 domain of PKCα (Guerrero-Valero et al., 2009; Verdaguer et al., 1999), PKCβ (Sutton and Sprang, 1998), PKCγ (Pike et al., 2007), PKCδ (Pappa et al., 1998), PKCε (Ochoa et al., 2001) and PKCη (Littler et al., 2006). These structures show that the C2 domain shares a common fold of conserved eight anti-parallel β-strands. β-strands are connected by variable surface loops. N-terminal region and surface loops are mostly variable among all these C2 domain structures. In classical PKC isoforms, three loops at the top of the β-strands provide all the residues which participate in calcium ions coordination. These three loops are referred to as CBR1 (calcium binding region), CBR2 and CBR3 (Fig. 2A). CRB1 corresponds to the β2 and β3 connection

loop which is conserved in all classical PKC-C2 (Fig. 2A), while it exhibits a large difference to novel PKC-C2 domains. CRB1 is replaced by loop1 (β1- β2) in novel PKC-C2 (Fig. 2B). Among all the PKC-C2 domains, the CRB3 structure (β6-β7) is highly conserved that corresponds to novel PKC-C2 loop3 (β5-β6). In contrast, the structure of the connecting loops at the end of the β-strand opposite the CRB is poorly conserved among the PKC-C2 domains.

The PKCβ-C2 domain coordinates with three calcium ions which are bonded through carboxylate groups from side chain and main chain atoms of CBR1 and CBR3, where CBR2 plays a supportive role (Sutton and Sprang, 1998). Three calcium ions binding sites were defined as site II, III and IV (Sutton and Sprang, 1998). Another classical PKC-C2 domain, the PKCα-C2 domain coordinates with two calcium ions (site II and III), while site IV was found to be occupied by one water molecule (Verdaguer et al., 1999). The structure of phospholipid bound PKC-C2-Ca^{2+} complex has also been resolved and this complex does not undergo significant conformational change upon phospholipid binding (Verdaguer et al., 1999). The C2 domain of PKCδ shares considerable sequence similarity with that of PKCθ. In contrast the PKCδ-C2 domain displays less than 20% sequence similarity with PKCε-C2 and PKCη-C2 domains. PKCδ-C2 domain also shows large structural differences with PKCε-C2 and PKCη-C2 domains. These differences include the presence in the PKCδ-C2 domain structure of a helix, between strands β6-β7 and of a protruding β hairpin (Ochoa et al., 2001). Loops connecting the β-strand

exhibit considerable variations within the novel PKC-C2 domains. Loop1 is the most variable and contains one α-helix near the N-terminal connection of PKCε-C2 and PKCη-C2 domains. In contrast, no α-helices are found in loop1 of the PKCδ-C2 domain or in the CRB1 of other classical PKC-C2 domains. Loop1 and loop3 correspond to the Ca^{2+} binding sites in classical PKC-C2 domain. These loops have shown to be essential for the interaction between the novel PKC-C2 domain and the phosphatidic acid (Ochoa et al., 2001). Moreover, recently the PKCδ-C2 domain has been characterized as phosphotyrosine binding domain that exhibited binding with diverse tyrosine phosphorylated proteins such as CDCP1 and PKD1 (Benes et al., 2005; Doppler and Storz, 2007).

PB1 domain: The PB1 domain is a protein interaction domain present in atypical PKC isoforms, adaptor and scaffold proteins as well as other kinases such as MEKK2, MEKK3 and MEK5 (Moscat et al., 2006). The PB1 domain is conserved in animals, fungi, amoebas, and plants that participates in diverse biological processes (Sumimoto et al., 2007). Crystallographic and NMR experiments have established the 3D structure of PKCι-PB1 domain (Hirano et al., 2004; Hirano et al., 2005). The PB1 domain contains two α-helices and a mixed β-sheet which contains five β-strands. This domain extends about 80 amino acid residues and binds to other PB1 domain containing proteins (Moscat et al., 2006).

PKC catalytic domain

PKC isoforms phosphorylate the substrate proteins on serine and threonine residues, usually in the context of a basic sequence (Newton, 2001) and catalytic domain alone is sufficient to complete this process. A bacterially expressed PKCθ catalytic domain was reported to be phosphorylated. The catalytic domain of PKC isoforms shows considerable (more than 60%) sequence similarity within in the family. Initially the structure of the catalytic domain of PKC isoform was determined by homology modeling based on the solved structure of PKA (Orr and Newton, 1994). PKA exhibits 40% of sequence similarity with PKC within the kinase domain. Recently the 3D structure of catalytic domain of several PKC isoforms such as PKCβ2 (Grodsky et al., 2006), PKCθ (Xu et al., 2004) and PKCι (Messerschmidt et al., 2005) has been resolved by crystallographic method.

The catalytic domain of PKC is folded into the classical bilobal fold as seen with other kinases. The smaller N-terminal lobe (N-lobe) is composed of five β-strands and two α-helices. The C-terminal lobe (C-lobe) is larger and is predominantly helical consisting eight α-helices. These two lobes are connected by a hinge-linker region. The active site cleft is built up by the ATP-binding site and the proximate peptide-substrate binding site and situated at the interface of both lobes. This site is solvent accessible. The glycine-rich phosphate-binding loop (GXGXXA)

connects two β-strands (β1-β2) of the bottom of N-lobe. The structure of the activation loop is well conserved across the PKC family with conserved threonine residue.

The C-terminal hydrophobic motif (FXXFS) is another conserved feature within the PKC family. Residues of this motif interact with the hydrophobic groove of the N-lobe. The serine residue of this motif is an autophosphorylation site. Atypical PKC isoforms have a glutamate at the site respective to serine (FXXFE). The glutamate residue of atypical PKC isoforms may act as phosphorylation mimic.

PKC activation

The activation mechanism of PKC isoforms has been studied extensively. The general mechanism of PKC activation is that PKC isoforms undergo a process of maturation before the enzyme is able to become activated (Fig. 3) (Newton, 2003). Newly synthesized PKCs move to the membrane and are phosphorylated by PDK1 in the activation loop which has the docking site in the hydrophobic motif (Sonnenburg et al., 2001). Activation loop phosphorylation is followed by two ordered autophosphorylations in the turn motif and hydrophobic motif (Newton and Koshland, 1987). These three phosphorylation sites are well conserved within the entire PKC family, excluding a carboxy-terminal hydrophobic site that is replaced with a glutamic acid residue in the atypical PKC isoforms. Mutation studies suggest that the turn motif

phosphorylation precedes the phosphorylation of hydrophobic motif (Edwards et al., 1999). These phosphorylations lead to stabilization and maturation of the PKC isoforms. However, the maturation mechanism is common for the classical PKC isoforms (Newton, 2003), the regulation of the hydrophobic motif of the novel PKC isoforms is less understood. Recent studies have led to the conclusion that phosphorylation of the novel PKCδ at the hydrophobic motif is catalyzed by another kinase and PKCε hydrophobic motif phosphorylation can be catalyzed by itself or by another kinase (Cenni et al., 2002; England et al., 2001; Parekh et al., 1999; Rybin et al., 2003; Ziegler et al., 1999). This maturation process results in a number of key conformational changes allowing the N-terminal pseudosubstrate region to interact with the substrate binding site, thus releases the enzyme from the membrane to the cytosol. This conformation keeps the enzyme as inactive form until activated by second messengers such as calcium and/or DAG.

PKC modulators

The discovery of isoforms-selective modulators of PKC is crucial to understand the role of the individual isoforms in physiological and pathophysiological processes and to manipulate their function. PKC modulators include the small molecule PKC activators and inhibitors. The discovery of isoform specific PKC modulators is at present a largely unmet pharmacological need.

PKC activators: The binding of DAG to C1 domain activates classical and novel PKC isoforms and for that reason DAG analogs are widely used as PKC activators. Several DAG analog phorbol esters such as phorbol 12-myristate 13-acetate (PMA)/12-O-tetradecanoylphorbol-13-acetate (TPA), phorbol-12,13-didecanoate (PDD), phorbol-12,13-dibutyrate (PDBu) and phorbol-12,13-dibenzoate (PDBz), but not phorbol-12-tetradecanoate, phorbol-13-acetate and 4α-phorbol-12,13-didecanoate had shown to activate PKC (Castagna et al., 1982; Kikkawa et al., 1983). These PKC activating phorbol esters are potent tumor promoters. Intracellularly DAG can be produced by the metabolism of phosphatidylinositol (PI), thus phosphatidylinositol-3,4-biphosphate (Toker et al., 1994), phosphatidylinositol-4,5-biphosphate (Berridge and Irvine, 1984) and phosphatidylinositol-3,4,5-triphosphate (Toker et al., 1994) are also potent PKC activators. Cis-unsaturated fatty acids such as arachidonic acid, oleic acid, linoleic acid, linolenic acid, linoelaidic acid and docosahexaenoic acid, activate PKC isoforms and cooperate with DAG (Lo et al., 1994; Murakami et al., 1986; Murakami and Routtenberg, 1985; Shinomura et al., 1991). In addition, fatty acids display some selectivity for the activation of specific PKC isoforms. For example, classical PKC isoforms (Shinomura et al., 1991) and novel PKCε (Koide et al., 1992) were shown to be activated by arachidonic acid, but another novel PKC isoform PKCδ was found to be inhibited by the same fatty acid (Ogita et al., 1992). PKC activators

targeting the C1 domain are unable to activate atypical PKC isoforms. A cell permeable ceramide has shown to activate PKCζ in a dose dependent manner (Muller et al., 1995).

PKC inhibitors: Many compounds competent to inhibit PKC activity have been used extensively to probe a variety of biological mechanisms and as an approach to treat cancer, diabetes and autoimmune diseases. Most of them target the catalytic domain of the PKC isoforms, although some of them target the regulatory domain. Unfortunately, most of PKC inhibitors suffer from various degrees of nonspecificity, both within the PKC family itself and also towards other kinases. Many potent PKC inhibitors are less specific to the PKC isoforms and very often inhibit other kinases. Staurosporine was one of the first PKC inhibitors to be identified. It interacts with the catalytic domain of the enzyme. The catalytic domain is most conserved within all serine/threonine protein kinases, and it is now recognized that staurosporine inhibits a range of other protein kinases. No compound has been reported to be specific for only one PKC isoform. The derivatives of staurosporine and its analog bisindolylmaleimides show higher selectivity to specific PKC isoforms. Regulatory domain inhibitors show some specificity to PKC but still lack isoform specificity. Recent studies suggest that isoform-specific PKC inhibitors are potentially beneficial to the prevention or treatment of some cancers and diabetics. Ruboxistaurin (LY333531), a potent PKCβ inhibitor has been shown to reduce the development of diabetic

vascular complications in animal models (Joy et al., 2005). Two partially isoform-specific PKC inhibitors, UCN-01 and CGP41251 exhibited partial responses in the prevention of the progress of malignancies (Shen, 2003).

PKC phosphoregulators

Phosphorylation is a reversible mechanism that regulates diverse cellular functions. Phosphorylation of proteins by protein kinases turns on a variety of signaling cascades that can be terminated by the protein phosphatases which dephosphorylate proteins.

PKC kinases: Classical and novel PKC isoforms are incapable of being activated by DAG without post-translational phosphorylations which are initiated by the activation loop phosphorylation and followed by couple of autophosphorylations in turn motif and hydrophobic motif. Evidence has mounted recently that PDK1 is the upstream kinase which directly phosphorylates the activation loop of many PKC isoforms (Table 1) (Cenni et al., 2002; Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Lee et al., 2005). The fact that PDK1 is the upstream kinase for all PKC isoforms has been emphasized by the further findings that, phosphorylation by PDK1 stabilizes PKC isoforms (Balendran et al., 2000) and that several PKC isoforms directly interact with PDK1 (Le Good et al., 1998). Although this is the common

mechanism that activation loop phosphorylation is mediated by PDK1, some other reports addressed that PKCε is the upstream kinase for PKCδ activation loop phosphorylation (Rybin et al., 2007; Rybin et al., 2003). The turn motif and hydrophobic motif phosphorylations at serine residues are catalyzed by PKC itself (Newton, 2003) excluding PKCδ hydrophobic motif phosphorylation that is catalyzed by another PKC isoform, PKCζ (Ziegler et al., 1999). Hydrophobic motif phosphorylation of PKCε is debated. One report has suggested that PKCε hydrophobic motif phosphorylation is mediated by a heterologous kinase (Parekh et al., 1999). Moreover, recently PKCδ has been identified as the PKCε hydrophobic motif kinase in cardiomyocytes (Rybin et al., 2003).

Besides the serine and threonine phosphorylations, PKC isoforms are also phosphorylated in several tyrosine residues. For example PKCα and PKCβ1 is phosphorylated by Syk (Kawakami et al., 2003), and PKCθ is phosphorylated by Lck (Liu et al., 2000) in tyrosine residues. PKCδ is in fact phosphorylated by various non-receptor tyrosine kinases, such as Abl, Fyn, Lck, Lyn and Src (Denning et al., 1996; Konishi et al., 2001; Sun et al., 2000; Szallasi et al., 1995). In contrast, classical PKC isoforms, such as PKCα, PKCβ and PKCγ, were not phosphorylated by src or only to a very small extent (Gschwendt et al., 1994). PKCζ has been identified to interact with Src (Seibenhener et al., 1999) and PKCι has shown to be tyrosine phosphorylated by the same kinase (Wooten et al., 2001). Atypical PKC isoforms are likely to be

activated by tyrosine phosphorylation which leads to the membrane translocation of PKCι (Wooten et al., 2001). Other known protein kinases which phosphorylate PKC isoforms include casein kinase-1 (CK1) and casein kinase-2 (CK2). Rat brain PKC was shown to be phosphorylated by CK1 (Vila et al., 1989). Another report demonstrated that PKCβ was phosphorylated by rat lung CK2 and that neither PKCγ nor PKCα was significantly phosphorylated by the same kinase (Tominaga et al., 1991).

PKC phosphatases: The dephosphorylation of proteins is catalyzed by three families of protein phosphatases. The serine/threonine phosphatase family members PP1 and PP2A are the major serine/threonine phosphatases in the cells. A heterotrimer form of PP2A dephosphorylated PKCα in vitro (Hansra et al., 1996) and inhibitors to PP2A have been shown to induce hyperphosphorylated forms of PKCα, PKCδ and PKCε (Gatti and Robinson, 1997). The purified PP2A, PP2C and catalytic subunits of PP1 were able to dephosphorylate baculovirus-expressed PKCδ in vitro (Srivastava et al., 2002). Baculovirus-expressed PKCδ retain phosphorylated in T505, S643 and S662 sites. However, none of these phosphatases displayed any marked site specificity to the PKCδ phosphorylation sites, PP2A found to be more selective to the PKCδ when compared to the relative specific activities (Srivastava et al., 2002). Another study showed that treatment with tautomycin, but not okadaic acid resulted in a significant increase in basal

phosphorylation of PKCα and PKCβ2 in MCF-7 cells (Kitatani et al., 2007). Tautomycin is an inhibitor for PP2A and PP1 which shows preference for PP1, but okadaic acid displays the opposite preference. This suggests that PP1 isoforms further identified as PP1cα and PP1cβ play a role in the control of PKCα and PKCβ2 phosphorylation in MCF-7 cells (Kitatani et al., 2007). Differential dephosphorylation of the PKCβ2 by catalytic subunits of PP1 and PP2A was explored (Dutil et al., 1994). PP1 dephosphorylates Thr500 plus Thr641 and Ser660 residues near the carboxyl terminus, yielding an inactive protein. PP2A dephosphorylates only Thr500 and Ser660 and yields a protein that can rephosphorylate itself to regain its native mobility (Keranen et al., 1995). Another study suggests that PKC isoforms interact with PP2A and retain as inactive kinase (Hansra et al., 1996). This interaction is abolished by the expression of a scaffold protein which binds to PP2A thus releasing PKC (Lee et al., 2008).

Conclusion

PKCs play key roles in mechanisms that regulate cell proliferation, survival, apoptosis and cell migration. PKCs are dysregulated in many clinically important disorders such as various immune disorders, atherosclerotic and diabetic cardiovascular diseases, and malignancies. The past few years have seen the identification of new key players which regulate PKC isoforms. Current PKC activation screening methods are quite limited. Most studies have relied on measurements

of PKC protein expression, which typically correlates at best only weakly with PKC activation. Measurements of PKC translocation to membranes are quite informative but translocation studies are not that convenient to use as screens for PKC activation. Understanding the mechanisms which control PKC activities will have important outcomes for potential therapies.

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Figure Legends

Fig. 1. Schematic representation of the structure of the PKC family.

Fig. 2. Three dimensional structures of PKC C2 domains.

Fig. 3. PKC activation mechanism.

Table 1. Isoform-specific PKC kinases

Group	Family	Kinase	PKC	Reference
AGC	PKB	PDK1	PKCα, PKCβ2, PKCδ, PKCε, PKCθ, PKCζ	(Cenni et al., 2002; Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Lee et al., 2005)
AGC	PKC	PKCδ	PKCε	(Rybin et al., 2003)
AGC	PKC	PKCε	PKCδ	(Rybin et al., 2003)
AGC	PKC	PKCζ	PKCδ	(Ziegler et al., 1999)
Other	CK1	CK1	Rat brain PKC	(Vila et al., 1989)
Other	CK2	CK2	PKCβ	(Tominaga et al., 1991)
TK	Abl	cAbl	PKCδ	(Sun et al., 2000)
TK	Src	Fyn	PKCδ	(Denning et al., 1996)
TK	Src	Lck	PKCδ, PKCθ	(Konishi et al., 2001; Liu et al., 2000)
TK	Src	Lyn	PKCδ	(Szallasi et al., 1995)
TK	Src	Src	PKCδ, PKCi	(Denning et al., 1996; Wooten et al., 2001)
TK	Syk	Syk	PKCα, PKCβ1	(Kawakami et al., 2003)

Figure 1.

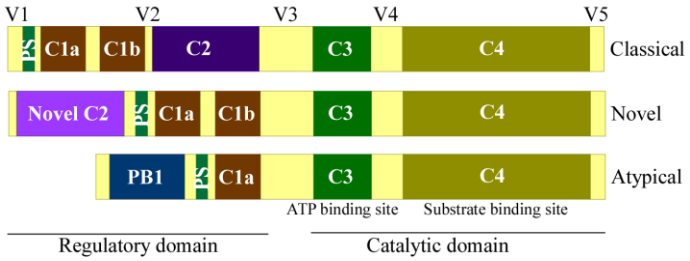

Figure 2.

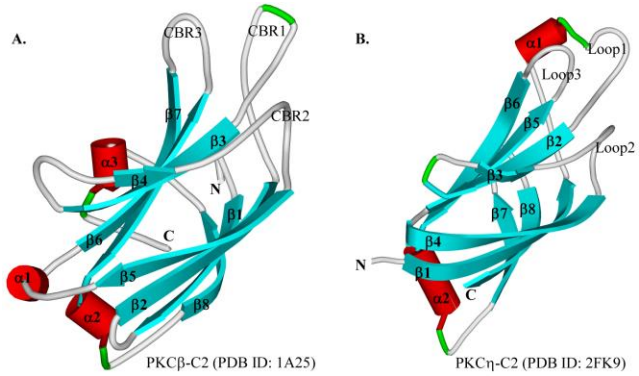

Figure 3.