NMR Structure/Function Relationships of Peptides Corresponding to the C1B1 Region of PKCγ

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Abstract: The region (101-112) of C1B domain in PKCγ plays a crucial role in the activation of the enzyme and subsequent gap junction inhibition. Substitution studies on peptides correlating to the C1B region show that a flexible structure and ability to be phosphorylated on serine 109 are critical for this purpose.

Keywords: Protein kinase C gamma, C1B domain, NMR, Structure function relationship, Serine 109.

INTRODUCTION

Protein kinase C (PKC) comprises a family of serine/threonine kinases which contain at least eleven isoforms and can be found in most cell types [1]. PKC’s can phosphorylate growth factor receptors, ion channels, structural proteins and gap junction connexin proteins [1, 2]. There are three isoform groupings of PKCs: classic, novel and atypical. PKCγ is a classical isoform and consists of four domains, see Fig. (1A). While C1 and C2 domains are regulatory, C3 and C4 are the catalytic domains [1]. PKCγ is a classic PKC but differs from other PKCs in that it does not need a calcium signal to be activated. This is because unlike the other classical PKCs, such as PKCα or PKCβ, the C1B domain is exposed while PKCγ is in the inactive state allowing direct access to the oxidation of the cysteine residue within the C1 domain [2, 3, 4].

The C1B domain has seven Cys residues that form a structure that contains a short α helix, five short β sheets and two zinc atoms [4, 5, 6]. The two zinc atoms create zinc fingers, the first one coordinating His101, Cys31, Cys34 and Cys150, the second one coordinating His138, Cys112, Cys117, and Cys142 [5]. In response to oxidative stress (H2O2), the six Cys residues and 2 His residues in the C1B domain are oxidized, the zinc is removed, and the Cys residues form disulfide bonds, causing changes in the protein conformation. This causes PKCγ to translocate to the plasma membrane [4]. PKCγ binds to the plasma membrane through the insertion of the hydrophobic tip (residues 120-125) into the cell membrane [5, 6]. Translocation is simply the binding of the hydrophobic tip of the C1B domain to the plasma membrane through hydrophobic interactions with membrane diacylglycerol (DAG), or its analogs, with high affinity. The hydrophobic tip consists of 6 hydrophobic amino acids that will insert ~10 Å into the membrane [6]. This region lies in the C1B region as can be seen in Fig. (1). Once the hydrophobic tip is trapped between the 2 fatty acid chains of DAG, it is said to be “bound” to the membrane [4].

Prior to insertion of the hydrophobic tip into the membrane, PKCγ must, first, be phosphorylated by 3-Phosphoinositide Dependent Protein Kinase-1 (PDK1). PDK-1 docks at the C terminus and phosphorylates PKCγ at the activation loop at Thr514 [1]. Upon phosphorylation of the activation loop, the active site is opened and PKCγ auto-phosphorylates at Thr541 [1]. Once this occurs PKCγ is released from PDK-1 and moves into the cytosol where it binds to a docking protein such as 14-3-3.

Another key to activating the enzyme once it has bound to the membrane is the removal of the pseudo-substrate. This is, in fact, the definition of activation. This event cannot occur on its own, however, the separation of the regulatory domains from catalytic domains is needed to pull the pseudo-substrate out of the active site. The creation of physical distance is the key to how this works. The binding of DAG and the protein’s C1 and C2 domain’s with the cytosolic side of the plasma membrane forces a conformational change in the structure of the protein [1]. This change in shape forces the removal of the pseudo-substrate from the active site. Once the active site is open, PKCγ auto-phosphorylates, becomes active [1], and then phosphorylates targets.

The C1B region of PKCγ is particularly important for regulation. It contains the consensus sequence for the 14-3-3 docking protein, the hydrophobic tip for insertion into the membrane and many positively charged residues for association with the negatively charged phospholipids of the plasma membrane. It also contains the two zinc finger regions mentioned earlier that are sensitive to oxidative stress.

There are two ways to activate PKCγ. One is to disrupt its association with its docking protein 14-3-3, the other is to alter the equilibrium of the cytosolic and membrane bound fractions. There are several known activators of PKCγ, such as oxidative stress which is often simulated with the addition of hydrogen peroxide (H2O2). Oxidation with H2O2 causes a conformational change in the C1B region of PKCγ by oxidizing the Cys residues and releasing the zinc ions. This change in shape frees PKCγ from its docking protein. An increase in the concentration of DAG in the membrane is also an activator. This is often simulated with a phorbol-
ester, such as Phorbol-12-Myristate-13-Acetate (TPA). This increase in DAG or phorbol ester in the membrane forces the translocation of PKCγ leading to its activation [3], see Fig. (8).

The docking protein which holds PKCγ in an inactive form is 14-3-3. 14-3-3 proteins comprise a group of acidic proteins of about 29-33 kDa [7]. 14-3-3s are found in all eukaryotic cells, and interact with over 300 proteins [8]. There are seven isoforms of 14-3-3 in mammalian cells, β, ε, γ, η, σ, θ, ζ [9,10]. 14-3-3 is most abundant in the cytoplasm [3].

The C1B1 peptide is a 12 amino acid synthetic peptide that follows the sequence of the first 12 amino acids of the C1B region of PKCγ, see Fig. (1C). Found on this peptide is the consensus sequence for 14-3-3: RSXpSXP. Previously, C1B1 peptide has been used to activate PKCγ, inhibit gap junction activity, and compete 14-3-3 off of known substrates [3, 11].

It has been shown previously that C1B1 inhibits gap junction activity. C1B1 competes PKCγ off of 14-3-3 increasing the probability for the activation of PKCγ. This enzyme activity has been proven to increase with the addition of C1B1 [3]. It has been shown that C1B1 decreases the amount of gap junction plaques in cells [3]. A decrease in gap junction plaques is often caused by in an increase in PKCγ activity through a PKCγ catalyzed phosphorylation on the C-terminus of gap junction proteins [12]. C1B5 has also been shown to compete 14-3-3 off of PKCγ [3]. In an experiment to determine a concentration curve for C1B peptides, Nguyen et al. determined that C1B5 competes PKCγ off of 14-3-3 in a concentration dependent manner [3].

The primary goal of this study was to prove the existence of a structure/function relationship in the binding of PKCγ and 14-3-3. This was shown by using functional data from live cells, in vitro affinity assays, in vivo affinity assays and Nuclear Magnetic Resonance (NMR).

MATERIALS AND METHODS

Reagents

N/N1003A rabbit lens epithelial cells were obtained from Dr. John Reddan (Rochester, MI). Polyclonal antibodies against 14-3-3 ε, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody against Rabbit antibodies was purchased from Pierce (Rock Ford, IL). Dulbecco’s modified Eagle’s medium (DMEM) (low glucose), trypsin-EDTA, gentamicin, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Anti-GST Agarose Beads were purchased from Invitrogen.

Synthetic Peptides

C1B1 sub-domain of PKCγ, residues 101–112 was synthesized by Biosynthesis Inc. (Lewisville, TX). Synthetic peptide C1B1 sequence is HKFRLHSSPT. Three point substitutions of this peptide were also synthesized at 95% purity, these are called M1, M2 and M3, see Fig. (1C).

Scrape loading/Dye Transfer

N/N 1003A cells were grown to 90% confluency on glass coverslips [11]. Cells were starved for 4 hours in low glucose DMEM without FBS. After 4 hours they were treated with

Figure 1. (A) Domains and regions of PKCγ. (B) C1B region of PKCγ in a linear sequence with the hydrophobic tip (solid box) and consensus sequences for docking with 14-3-3 (dotted boxes) identified. (C) C1B1 peptides with locations of substitutions in bold.
one of four 12 amino acid peptides, each at 100μM, in water. These peptides, synthesized by Biosynthesis at 95% purity, were the C1B1 region of PKCγ and 3 point mutations of that peptide. The sequences are listed in Fig. (1). Peptides were incubated for 2 hours or cells were given 200 nM TPA for 20 minutes as a positive control. It has been shown previously by mass spectrometry that ~27% uptake of C1B1 in N/N 1003A was noted after 2 hours [3]. TPA is a known activator of PKCγ [3]. This is used to demonstrate a 100% active enzyme at 200nM in N/N 1003A cells. A 5 μL drop of a mixture of 2% each Lucifer Yellow and Rhodamine Dextran were added to the cells at the center of the coverslip. 2 cuts were made across the center of the coverslip in the shape of an X to form a transient tear in the plasma membrane of the cells to permit dye transfer through the gap junctions. Dye was allowed to permeate the cells for 2 minutes, and then rinsed away with PBS. Rhodamine Dextran is a dye that does not pass through gap junctions, while Lucifer Yellow does. For this experiment cells with red dye were damaged while those with only yellow showed gap junction activity. Cells were then incubated with the dye and DMEM with FBS for 20 minutes, then fixed in 3% paraformaldehyde and examined by fluorescent microscopy. Each time the experiment was conducted, each treatment was conducted on three coverslips, and pictures of three fields from each coverslip were taken. The number of cells to which dye was transferred (green) was divided by the number of damaged cells (red). This gives an average number of cells to which dye was transferred per cell over a 20 minute time period.

**Binding Assays**

**In Vivo**

N/N 1003A cells were treated for 4 hours in DMEM media with 100μM peptides or no peptide for the control. The cells were then harvested and lysed in 1mM Tris.HCl (pH 7.4). Immunoprecipitation was performed to “pull down” PKCγ. The samples were then centrifuged at 1200 rpm in a Labnet Z-233 Mk-2 centrifuge at 4°C. In order to limit the background from non-PKCγ bound 14-3-3, we used the agarose beads for determining affinity. The beads were treated with 20 μL of 2X loading buffer (62.5 mM Tris-HCl, 20% Glycerol, 2%SDS, pH6.8) with DTT at 100 μL drop of a mixture of 2% each Lucifer Yellow and Rhodamine Dextran were added to the cells at the center of the coverslip. 2 cuts were made across the center of the coverslip in the shape of an X to form a transient tear in the plasma membrane of the cells to permit dye transfer through the gap junctions. Dye was allowed to permeate the cells for 2 minutes, and then rinsed away with PBS. Rhodamine Dextran is a dye that does not pass through gap junctions, while Lucifer Yellow does. For this experiment cells with red dye were damaged while those with only yellow showed gap junction activity. Cells were then incubated with the dye and DMEM with FBS for 20 minutes, then fixed in 3% paraformaldehyde and examined by fluorescent microscopy. Each time the experiment was conducted, each treatment was conducted on three coverslips, and pictures of three fields from each coverslip were taken. The number of cells to which dye was transferred (green) was divided by the number of damaged cells (red). This gives an average number of cells to which dye was transferred per cell over a 20 minute time period.

**In Vitro**

N/N 1003A cells were harvested and lysed in cell lysis buffer. Immunoprecipitation was performed to pull down PKCγ in the same cell lysis buffer. The agarose beads were then rinsed with the same buffer 3 times. 500 μL of buffer was then added and 100 μM of C1B1 or each of the 3 mutants was added. The samples were mixed thoroughly for 2 hours at 4°C, and then were centrifuged at 1200 RPM at 4°C. In order to limit background interference the supernatant was used for the samples. The supernatant was removed, 5X loading buffer was added, boiled and loaded on a gradient gel as described above. The blot was treated with Rabbit anti-14-3-3-ε antibodies from Santa Cruz at a concentration of 1:100 in 5% milk overnight at 4°C. The blot was then rinsed in TDN (1 M NaCl, 0.2 M Tris base, and 0.04 M EDTA, pH 7.4) and treated with goat anti-Rabbit secondary antibody from Pierce in 5% milk for 1.5 hours at room temperature. The blot was then treated with super substrate from Pierce for 5 minutes and exposures were taken for varying times. The film was scanned into Un-Scan-It software and the relative amounts of free 14-3-3 in each sample were determined by total average pixel intensity.

**Cell Viability Assay**

Cell viability assay was performed using CellTiter-Blue® Cell Viability Assay kit (Promega Madison, WI). The kit applies a fluorometric method for measuring the number of viable cells in 96 well plates. Viable cells have the ability to convert redox dye resazurin into product resorufin which is highly fluorescent (560/590 Ex/Em). Therefore, the amount of fluorescence produced is directly proportional to the number of viable cells. Nonviable cells cannot reduce resazurin and therefore do not produce any fluorescent signal. For this assay, 30 × 10^3 N/N 1003A cells in 100μL of DMEM low glucose medium were seeded at each well in 96 well micro-titre plates and cultured for 12 hours at 37°C. The cells were then treated with 50 μM, 100μM and 200μM concentration of each peptide or no peptide and no H₂O₂ for the control and incubated for 4 hours. After that, the cells were incubated with 100μM H₂O₂ for 12 hours. For the estimation of number of viable cells, 20μL of CellTiter-Blue reagent was added in each well and following incubation of 4 hours fluorescence was recorded at 560(5) Ex/590(5) Em. The assay was conducted in four replicates and average values were graphed in Fig. (5).

**Statistics**

The dye transfer assays were all conducted at least in triplicate. The results were averaged based on the number of cells transferred to, and the control was given a 100% transfer rate. The treatments are expressed as percentages of inhibition. All affinity assays were conducted a minimum of 3 times. The results were normalized to the control and averaged NMR statistics were computed using the program CNS which computes all the statistics reported in Table 1.

**NMR**

A series of 1D and 2D [1H-1H] NMR experiments for the wild type 12-residue C1B1 peptide and 3 other peptides containing single amino acid substitutions were performed as previously described [21]. Briefly, NMR data were acquired on an 11.75T Varian UNITYplus spectrometer (Varian, Palo Alto, Ca) operating at 500 MHz for 1H with a 3-mm triple resonance inverse detection probe. The NMR sample consisting of 4mM peptide concentration in water containing 50% deuterated TFE (TFE-d₃, Cambridge Isotope Labora-
Table 1. Structure Statistics Table

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 RESULTS

Effect of C1B1 and Mutants on Gap Junction Activity

Gap junctions between N/N 1003A cells are controlled by PKCγ. The activation of PKCγ causes phosphorylation of connexins and the closing of gap junctions [3, 12]. In order to test the degree to which C1B1 inhibits gap junction activity, the scrape loading/dye transfer technique was used. The peptides were used at 100μM and the tumor promoting agent 12-O-tetradecanoyl-phorbol-13-acetate, or phorbol 12-myristate 13-acetate (TPA) was used at 200nM as a positive control. Cells treated with C1B1 or M1 showed inhibition of gap junctions. Fig. (2A) and (2B) shows that C1B1 and M1 decrease the amount of dye transfer in N/N1003A cells by 40%. This is comparable to TPA, the known activator of PKCγ. Cells treated with M2 and M3 showed no inhibition of dye transfer.

In Vitro Competition Binding Assay

The in vitro assay was conducted on co-immuno-precipitated PKCγ and 14-3-3e. The in vitro assay demonstrates how much 14-3-3e was competed off of PKCγ. The
Figure 2. Dye Transfer. N/N 1003A cells were starved for 4 hours then 100μM C1B1 or one of the 3 mutant peptides was added to the cells and allowed to incubate for 2 hours. 200 nM TPA for 20 minutes was used as a positive control. Dye was allowed to permeate the cells for 2 minutes, the cells were washed in PBS, and dye transfer proceeded for 20 minutes. (A) Quantitative analyses from three different experiments are graphed and the error bars represent standard error. (B) Photographs of dye transfer showing the difference between control where dye transfers to many cells and C1B1 and TPA where dye transfers very little.

Figure 3. In Vitro affinity assay. N/N 1003A cells were harvested and lysed then immunoprecipitation was performed to pull down PKCγ. The samples were then treated for 4 hours with 100μM peptides or no peptide for the control. The supernatant was removed and loaded on to a gradient gel and western blotted using anti 14-3-3ε as per the methods. The images were scanned and total average pixel intensity was recorded. The results of four scans were normalized, averaged based on a control value of 1 and graphed. Quantitative analyses from four different experiments are presented and the error bars represent standard error.

greater the signal in Fig. (3), the more effective the peptide was at competing 14-3-3 off of PKCγ. Samples treated with C1B1 and M1 showed a 2x signal as compared to the control. M2 was nearly as effective at 1.7 times the control. There was a greater ability of M2 to compete off 14-3-3 than its ability to alter dye transfer. Samples treated with M3 still had no effect, when compared to the control with no peptide added.

Since these results differed from the results seen with dye transfer, an in vivo binding/competition assay was completed to determine if this was due to in vitro vs. in vivo differences.
In Vivo Competition Binding Assay

To test whether cellular modification of C1B1 and its mutants was needed to achieve the same affinity results as was seen with dye transfer, C1B1 peptides were added directly to cells in culture. In order to limit the background from free cellular 14-3-3, the immuno-precipitated beads were used to determine affinity. In Fig. (4), a higher signal means less 14-3-3 competed off of PKCγ, and the less effective the peptide at activating PKCγ. The results of the in vivo assay are in agreement with the dye transfer assay. Once again, the M1 peptide was equally as effective as the parent, C1B1.

Cell Viability Assay

In order to further investigate the effect of C1B1 and mutant peptides on gap junction activity, we performed cell viability assay. For this purpose, 1003A N/N cells were treated with 50μM, 100μM and 200μM of each peptide for four hours. Then 100μM of H2O2 were added for 12 hours to each sample in order to produce oxidative cell death. In Fig. (5), a notable decrease in viability was observed in case of cells pre-treated with mutant M2 and M3 peptides. But the cells which were pretreated with C1B1 or M1 peptides remained viable to a considerable extent even after H2O2 treatment. Thus C1B1 and M1, but not M2 and M3, were able to protect the cells from death caused by H2O2.

Structural NMR Studies

For structural calculations, NOE derived distance restraints were classified into four ranges: 1.8-2.7, 1.8-3.5, 1.8-4.0, and 1.8-5.0Å, according to strong, weak and very weak NOE intensities. Upper distance limits for NOEs involving methyl protons and non-stereospecifically assigned methylene protons were corrected appropriately for center average [13]. In addition, a distance of 0.5Å was added to the distance limits only for NOEs involving the methyl proton after correction for center averaging [14]. The distance restraints were then used to create initial peptide structures starting from extended structures using the program CNS (version 1.1) [15]. CNS uses both a simulated annealing protocol and molecular dynamics to produce low energy structures with the minimum distance and geometry violations. In

Figure 4. In vivo Affinity assay. N/N 1003A cells were treated for 4 hours with 100μM peptides or no peptide for the control. The cells were harvested and lysed then immunoprecipitation was performed to pull down PKCγ. The supernatant was removed and the beads were washed in SDS sample buffer and the eluted proteins were run on a gradient gel and western blotted with anti-14-3-3ε as per methods. The images were scanned and total average pixel intensity was recorded. The results of four scans were normalized, averaged and graphed setting the control value of 1. Quantitative analyses from four different experiments are presented and the error bars represent standard error.

Figure 5. Cell Viability Assay: 30 × 10^3 N/N 1003A cells were treated for 4 hours with 50μM, 100μM and 200μM of each of the four peptides. Then the cells were incubated with 100μM H2O2 for 12 hour. Cells treated with no peptide and no H2O2 were used as control. Following H2O2 incubation, cell viability was analyzed by resazurin reduction method as described in the Methods Section. The plots represent the average of results obtained in four replications of the assay.
general, default parameters supplied with the program were used with 100 structures for each CNS run. The final round of calculations began with 100 initial structures and 20 best structures with the lowest energy selected and analyzed with MOLMOL [16] and PROCHECK-NMR [17]. Structure figures in Fig. (6) were generated using MOLMOL.

The average structures of C1B1, M1 and M2 predict a random structure. They appear to have the same general shape, Fig. (6). A superimposition of the 20 lowest-energy structures showed a considerable degree of flexibility, in C1B1 and M2, with pairwise RMSD of the backbone of 3.27Å, and 2.86Å respectively. M1 showed less flexibility with a RMSD of 2.47 Å and M3 showed the least amount of flexibility with an RMSD of 2.10Å (Supplemental Fig. (S1) and Table 1).

In order to see how these structures compared to the structure found in the actual protein, the published NMR structure of the entire C1B domain of PKCγ from the RCSB protein data bank was used. By only selecting the residues we were concerned with (102-113, 1 residue off from our numbering system), the original sequence structure and the NMR structures of the peptides were lined up, see Fig. (7). Also note the orientation of the serine side chains (circled in each figure). It is apparent that the Ser9 is displaced in M2 and especially in M3.

**DISCUSSION**

There are two different means of regulation for the translocation of PKCγ: 1) when the structure of PKCγ is changed due to oxidative stress, the proteins disassociate. 2) When there is an increased concentration of DAG in the membrane, the non-docked PKCγ binds to the membrane. These two events bring about the translocation of PKCγ in different ways. In the first event, the dissociation constant of PKCγ for 14-3-3 increases due to a change in structure, and this increase in dissociation allows for PKCγ to have a greater probability of binding to DAG in the membrane. This change in structure is where the 30Å between the two consensus sequences of 14-3-3 is shifted. In the second event, there is no increase in the disassociation constant of PKCγ for 14-3-3, but there is an increase in the probability of PKCγ binding DAG due to the increase in membrane DAG, see Fig. (8). It is the ability of DAG to reach the hydrophobic tip of the PKCγ C1B domain that controls the extent of PKCγ translocation and activation.

How does the C1B1 peptide cause the activation of PKCγ? The peptide acts in a similar manner to oxidative stress in that it interrupts the binding of 14-3-3 and PKCγ. It does not however, oxidatively alter the zinc finger motif of the C1B region. It simply displaces the entire protein from 14-3-3, allowing association with the plasma membrane, interaction with and inhibition of gap junctions, Fig. (8).

![Figure 6](image-url). NMR structures of the 12-residue C1B1 peptide which corresponds to residues 101-112 of PKCγ, and 3 peptides containing single amino acid substitutions labeled M1-3 respectively. NMR structures of all four peptides individually (A-D) and overlaid (E). In all figures C1B1 is colored orange, M1 is colored grey, M2 is colored green, and M3 is colored blue.
Figure 7. NMR structure of the native sequence of C1B1 region from the structure of the C1 domain of PKC\(\gamma\) from Xu et al. 1997 [5] (1TBN.PDB), compared to the peptides. The Ser\(^9\) and Ala\(^9\) side chains have been visualized and circled. A. C1B1 and the original sequence. B. M1 and the original sequence. C. M2 and the original sequence. D. M3 and the original sequence. The wild type C1B1 protein is colored yellow.

Nguyen et al. determined the effects of the C1B1 peptide on activation of PKC\(\gamma\) and on the binding of PKC\(\gamma\) and 14-3-3 [3]. This study attempted to expand on those results by using single amino acid substitutions of the C1B1 peptide, see Fig. (1C). Substituting a tyrosine for a phenylalanine in mutant 1 (M1), would determine if tyrosine phosphorylation was required at position 8. By substituting a serine for an alanine at position 9 in mutant 2 (M2), the effects of removing the known phosphorylation site Ser\(^9\) could be determined [18-20]. This phosphorylated serine, 9 in the peptide and 109 in PKC\(\gamma\), may allow for hydrogen bonding between 14-3-3 and either the peptide or the protein. The energy supplied by the hydrogen bond could allow for differences in binding affinities. Our results support this hypothesis.

Finally, by changing the histidine to a phenylalanine at position 6, the effects of the loss of a charge on the peptide at this position could be determined. Histidine can be charged or uncharged based on the pH. In PKC\(\gamma\) His\(^{106}\) is in a position of the 14-3-3 consensus sequence normally occupied by an Arg (RSXSXP) [18]. For this reason we believe the histidine is charged, and the charge is important to structure.

By using these 3 mutations we have determined the phosphorylation dependence and structural dependence of the peptide.

A greater understanding of the structure function relationship of the C1B1 peptides and the ability to activate PKC\(\gamma\) could lead to drug development. These drugs could lead to the delay of the neuro degenerative disease spinocerebellar ataxia type 14 (SCA14). In this disease, mutant PKC\(\gamma\) fails to control gap junction communication leading to neuro-degeneration. The mutations lead to the death of purkinje cells. This degeneration is due to what is called the “by-stander effect”, where death signals are passed from damaged cells to neighboring cells through poorly controlled gap junctions [2]. We have found that the C1B1 peptide restores normal PKC \(\gamma\) activity in HT22 cells which are overexpressing PKC \(\gamma\) with SCA-14 mutations [22].

Here, our study shows that there is a structure/function relationship between the consensus sequence of PKC\(\gamma\) and the binding site of 14-3-3. By using different amino acid substitutions within the 12 amino acid sequence that is analogous to the C1B1 region of PKC\(\gamma\) we have shown that a specific structure and the ability to phosphorylate at Ser\(^{109}\) are key to the interaction. Fig. (2) shows that the C1B1 pep-
Figure 8. Possible mechanism of dissociation of PKC\(\gamma\) and 14-3-3. (A) Inactive PKC\(\gamma\) is bound to 14-3-3 in the cytosol of cells. This equilibrium is shifted to the left in the diagram above. When an event occurs to activate PKC\(\gamma\) (oxidative stress) it shifts this equilibrium more to the right. DAG binds the C1B region of PKC\(\gamma\) and holds it with a high affinity to the membrane. When an event occurs that shifts the first equilibrium to the right, it allows more PKC\(\gamma\) to associate with DAG in the membrane. An increase in available DAG can also shift the entire equilibrium to the right causing activation of PKC\(\gamma\). (B) It has been said that H\(_2\)O\(_2\) acts like DAG in activating PKC\(\gamma\). This model shows that although they have the same result, there is a very different mechanism. H\(_2\)O\(_2\) alters the equilibrium between 14-3-3 and PKC\(\gamma\). Increase in DAG alters the equilibrium between free cytosolic PKC\(\gamma\) and membrane bound PKC\(\gamma\). (C) C1B1 alters the equilibrium between 14-3-3 and PKC\(\gamma\) by direct competition without altering the conformation of PKC\(\gamma\).

Peptide inhibits gap junction activity to the same extent as TPA which is in agreement with results reported by Lin, et al. 2007. Our results also show that M1 has the same efficiency as C1B1. M2 and M3 do not inhibit gap junction activity at 100\(\mu\)M.

The inhibition of gap junction activity by C1B1 and mutant M1 peptide were further demonstrated by cell viability analysis. Cells treated with C1B1 and M1 peptides were protected from death by H\(_2\)O\(_2\) but cells treated with M2 and M3 were vulnerable to H\(_2\)O\(_2\). Fig. (5). It suggests that the inhibition of gap junctions by C1B1 and M1 peptides restricts the passage of "stress" signal from damaged cells to neighboring cells keeping the neighboring cells alive.

Why does M3 fail to compete 14-3-3 off of PKC\(\gamma\) while M2 has limited ability and M1 and C1B1 show ability to do so? The answer to this question lies in the structure. As can be seen from the NMR structures of all of the peptides, C1B1, M1 and M2 have a loose structure, while M3 has a more organized structure, see Fig. (6) and Fig. (S1). There is a helical region, from residues 4-7, that is not present in C1B1, M1 and M2. There is also a loop that brings Ser\(^9\) close enough to Lys\(^5\) (i+1) that may prevent phosphorylation from occurring \textit{in vivo}. This could account for the differences seen in the dye transfer and \textit{in vivo} affinity assays. This helical region, with the loop that follows would prevent the peptide from aligning properly with the binding site on 14-3-3. Failure to insert into this site could prevent M3 from displodging 14-3-3 from PKC\(\gamma\).

M2 shows limited ability to compete 14-3-3 from PKC\(\gamma\). M2 showed this ability to compete \textit{in vitro}, where there is not a possibility of phosphorylation. Therefore this ability may be due to the structure. Its structure is most like the structure of the native PKC\(\gamma\) C1B1 sequence Fig. (7), giving it the advantage over M1 and C1B1.

Another interesting result was found from M1, in which tyrosine was changed to phenylalanine. Results support the literature indicating that there is no difference in the ability of M1 to disrupt the binding of PKC\(\gamma\) to 14-3-3 and therefore no dependence on tyrosine phosphorylation.

The future use of C1B1 peptides could help to activate PKCs which are inhibited in genetic diseases such as Spinocerebellar ataxia type 14 (SCA 14). SCA14 is a neurodegenerative disease caused by a mutation in the PRKCG
gene. In this disease, mutant PKC\(\gamma\) fails to control gap junction communication leading to neurological degeneration. The mutations lead to the death of purkinje cells. This degeneration may be due to what is called the "bystander effect", where death signals are passed from damaged cells to neighboring cells through poorly controlled gap junctions. SCA 14 is characterized by a dominant negative effect and wild type PKC\(\gamma\) is somehow prevented from performing its function by the mutated form of PKC\(\gamma\) [11, 22]. It is believed that if you could find a way to prevent the dominant negative effect, you could delay the effects of ataxia until much later in life.

With proper understanding of the physical mechanism by which the C1B region of PKC\(\gamma\) binds to 14-3-3 we hope to synthesize peptides with increased ability to activate PKC\(\gamma\) and control gap junctions. This could eliminate the dominant negative effect and limit the bystander effect, delaying the effects of ataxia until much later in life.

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ABBREVIATION

PKC\(\gamma\) = Protein kinase C gamma
C1B = The lipid-binding domain of PKC

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

REFERENCES