NMR structure/function relationships of peptides corresponding to the C1B1 region of PKCγ

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Purpose: PKCγ is a serine/threonine kinase that inhibits the activity of membrane gap junctions in response to oxidative stress. In the absence of oxidative induction, PKCγ is held in an inactive state in the cytoplasm by docking to 14-3-3 proteins at the C1B regulatory domain of PKCγ. The purpose of this study is to prove the existence of a structure /function relationship in the binding of PKCγ to 14-3-3 through its C1B domain.

Methods: Synthetic peptides of 12 amino acids in length corresponding to the C1B1 region (101-112) of PKCγ were tested for our studies. The peptides were wild type and three mutants M1 (Y108F), M2 (S109A) and M3(H106F) C1B1 peptides. The gap junction activity scrape loading /dye transfer assay was done on 90% confluent N/N 1003A cells pre-treated with 100uM of C1B1 or each of the three mutants for 2 hours.. Cell death analysis was performed by a fluorometric method on 30 × 103 N/N cells pre-treated with 50uM, 100uM and 200uM concentration of each of the four C1B peptides followed by the treatment with 100uM H2O2 for 12 hours. For structural determination of C1B1 peptides 1D and 2D 1H-1H NMR experiments were performed on a 5000MHz UNITY plus spectrometer at 10 degree C in H2O :TFE-d3 (1:1V/V).TOCSY and NOESY experiments were used to collect structural information of C1B1 peptides..

Result: Wild type C1B1 and mutant M1, but not M2 and M3, decreased the amount of dye transfer in N/N 1003A cells by 40% which indicated PKCγ activation and subsequent gap junction inhibition. Cells treated with C1B1 and M1 peptides were protected from death by H2O2 but cells treated with M2 and M3 were vulnerable to H2O2 .. NMR studies showed a flexible structure for all the peptides with the structure of M3 more organized than C1B1, M1 or M2.

Conclusion: The C1B1 domain of PKCγ binds to 14-3-3 protein. Upon oxidative stress, this interaction is disturbed and PKCγ becomes activated. Once activated, PKCγ phosphorylates and inhibits gap junction activity. There is a structure/function relationship between the consensus sequence of PKCγ (101-112) and the binding site of 14-3-3. The ability to be phosphorylated on the serine at position 109 is critical for the peptide to induce activation of PKCγ.

Wild type and mutant M1 (Y108F), M2 (S109A) and M3(H106F) C1B1 peptides

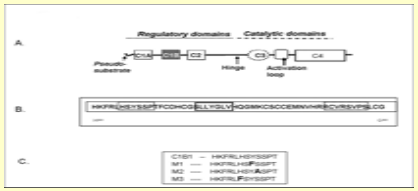

Figure 1:
A. Domains and regions of PKCγ.
B. C1B region of PKCγ in a linear sequence with the hydrophobic tip (solid box) and consensus sequences for docking with 14-3-3 (dotted boxes) identified.
C. C1B1 Peptides with locations of substitutions in bold.

Functional Studies

Scrape Loading/Dye Transfer: N/N 1003A cells were starved in DMEM media without FBS 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. A mixture of Lucifer Yellow and Rhodamine Dextran was added to the cells at the center of the coverslip. 2 cuts were made across the center of the coverslip to form a transient tear in the plasma membrane of the cells to permit dye transfer through the gap junctions. Cells were incubated with the dye and DMEM with FBS for 20 minutes, then fixed and examined by fluorescent microscopy. 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 damaged cell.

Cell Viability Assay: 30 × 103 N/N 1003A cells were treated for 4 hours with 50uM, 100uM and 200uM of each of the four peptides. Then the cells were incubated with 100uM H2O2 for 12 hour. Cells treated with no peptide and no H2O2were used as control. Following H2O2 incubation, cell viability was analyzed by resazurin reduction method in which the number of viable cells is directly proportional to the amount of fluorescence produced.

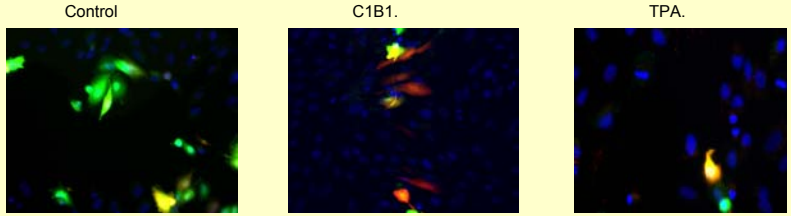

Figure 2. Pictures of Dye transfer showing that TPA completely inhibits gap junction activity, as does C1B1. In control, non-treated cells show extensive gap junction activity.

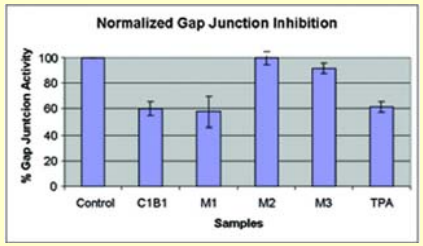

Figure 3. The effects of C1B1 peptide and three mutant peptides on dye transfer in NN1003 cells.

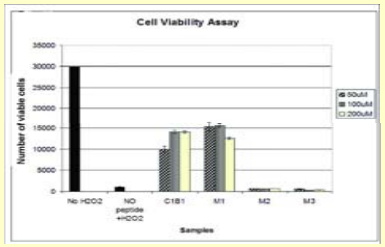

Figure 4. The protective effect of C1B1 peptides from death caused by H2O2

Results: Cells treated with C1B1 and M1 showed inhibition of gap junctions.C1B1 and M1 decrease the amount of dye transfer by 40% which is comparable to TPA, the known activator of PKC.The result of cell viability assay was in agreement with dye transfer assay. 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.

NMR Studies

NMR Methods: 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 on a 500 MHz Varian UNITYplus spectrometer at 10 °C in H_2O: TFE-d_3 (1:1 V/ V). TOCSY and NOESY experiments were used to collect structural information. Sparky software (Goddard & Kneller, 2004) was used for data analysis. Proton resonance assignments were made using 2D TOCSY for intra-residue spin systems and NOESY spectra for inter-residue connectivities by standard techniques. Distance constraints were used in the program Crystallography & NMR System (CNS) version 1.1 (Brunger et al., 1998) for structure calculation. CNS uses both a simulated annealing protocol and molecular dynamics to produce low energy structures with the minimum distance and geometry violations. A total of 200 conformers were simulated from an extended conformation and we chose 20 structures with minimal NOE violations. These 20 conformations are in good agreement with the experimental data, with no distance violations larger than 0.3 Å and no angle violations of more than 5°. The lowest total energy and then took the average of these to represent the three-dimensional NMR structure.

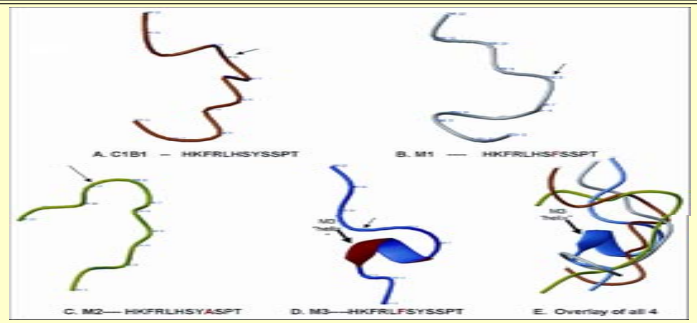

Figure 5: NMR structures of the 12-residue C1B1 peptide which corresponds to residues 101-112 of PKCγ, and 3 peptides containing single amino acid substitutions labeled M1-3 respectively. NMR structures of all four peptides individually (A-D) and overlaid (E). In all figures C1B1 is colored orange, M1 is colored grey, M2 is colored green, and M3 is colored blue .

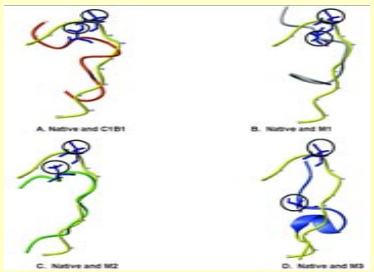

Figure 7: NMR structure of the native sequence of C1B1 region from the structure of the C1 domain of PKCγ from the RCSB protein data bank compared to the peptides. The Ser9 and Ala9 side chains have been visualized and circled. A. C1B1 and the original sequence. B. M1 and the original sequence. C. M2 and the original sequence. D. M3 and the original sequence. The wild type C1B1 protein is colored yellow

	C1B1	M1	M2	M3
Total NOE	157	124	135	100
Intra-residue	35	68	68	51
sequential	79	38	46	27
Medium	43	18	21	22
const/res	13.08	10.33	11.25	8.33
Energy (Kcal/mol^{-1})				
overall	26.54 +/-4.68	18.47 +/-0.42	40.64 +/-1.02	35.18 +/-2.01
bond	0.84 +/-0.16	0.46 +/-0.05	1.83 +/-0.21	1.11 +/-0.14
angle	14.57 +/-0.71	11.88 +/-0.23	18.83 +/-0.45	17.58 +/-0.87
improper	0.36 +/-0.08	0.36 +/-0.05	1.15 +/-0.20	3.16 +/-0.25
vander Waals	8.37 +/-4.64	5.07 +/-0.33	13.61 +/-0.84	9.26 +/-0.91
NOE	2.38 +/-0.76	0.71 +/-0.15	5.23 +/-0.72	4.07 +/-1.19
RMSD (Å)				
Backbone	3.27 +/-0.74	2.47 +/-0.70	2.86 +/-0.86	2.10 +/-0.82
Heavy	4.66 +/-0.88	3.39 +/-0.81	4.09 +/-1.11	3.12 +/-0.93

Table 1. Structural statistics for 20 conformations used to represent the solution structure of the C1B1 and mutant peptides.

Result of NMR studies: The average structures of C1B1, M1 and M2 predict a random structure. They appear to have the same general shape. A superimposition of the 20 lowest-energy structures showed a considerable degree of flexibility with the structure of M3 more organized than C1B1, M1 or M2. There is a helical region,from residues 4-7,in M3 that is not present in C1B1, M1 and M2.

Conclusions:

1. C1B1 peptide associates with 14-3-3 releasing PKCγ. PKCγ then becomes activated. Once activated it can phosphorylate Connexins thereby decreasing Gap Junction activity.

2. The tertiary structure of the C1B1 peptide takes a helical conformation in the Ser6-Ser9 region, while its N- and C- termini are flexible. The flexibility may facilitate the binding mechanism of C1B1 peptide to 14-3-3.

3. It can be postulated that the flexible structure of the peptide in its 12 amino acid form allows it to associate with 14-3-3 in a non hindered manner and releases PKCγ from its docked position in the cytosol allowing it to phosphorylate Connexins.

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