Lens PKCγ - C1B Domain is Altered by Ataxia Mutations : A Modeling NMR Study

D. Shanks, D. Madgwick, M. Barnett, D. Lin, O. Prakash, L. Grauer, D. Takemoto

Department of Biochemistry, Kansas State University, Manhattan KS, 66506

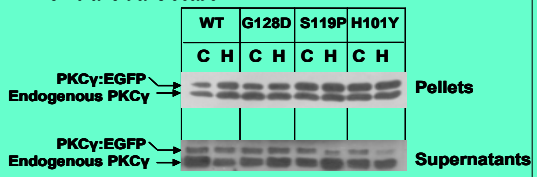
Abstract: There are a number of human diseases which are linked to the failure of proper gap junction control. Mutations in PKCγ C1B domain are associated with Human Spinocerebellar Ataxia, SCA-14. The mutations lead to an impaired cellular response to extracellular signals and oxidative stress. The purpose of this research is to determine the structural influence of C1B domains mutations on the functional activation of PKCγ, a major lens PKC.

Methods: N/N 1003A cells that were 80% confluent were stably transfected with pEGFPN3 plasmids containing PKCγ WT, H101Y, S119P, and G128D. The cells were selected in G418 DMEM media. To determine PKCγ enzyme activity the commercially available Peptag assay kit was used (Promega). For membrane translocation, cytosolic and membrane fractions were separated and probed with anti-PKCγ antibodies (BD). Analyses of the phosphorylation state of PKCγ and Cx43 were done with anti-pT514 and anti-pS368 antibodies (respectively).

Results:

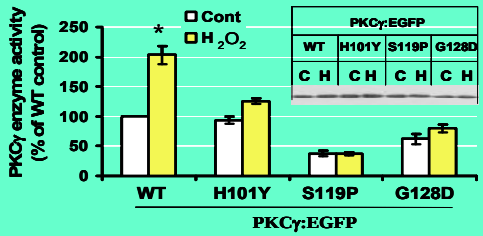
1. Membrane translocation experiments (Fig.A) demonstrate that the three SCA-14 PKCγ mutants are found in both pellet and supernatant fractions after H_2O_2 treatment.
2. Enzyme activity assays for PKCγ (Fig.B) show that the SCA-14 mutants are not activated when compared to WT.
3. Analysis of the autophosphorylation state of PKCγ Thr^{514} WT and mutants (Fig.C) demonstrate that specifically Thr^{514} is phosphorylated in response to H_2O_2 treatment while the mutants are not.
4. Analysis of the phosphorylation state of Cx43 (Fig.D) shows PKCγ WT phosphorylates Cx43 on Ser368 but the mutants do not.

A. Membrane translocation



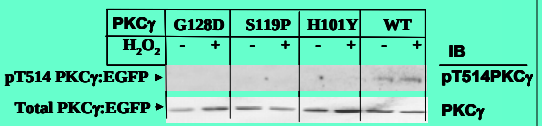
Membrane translocation was examined using confluent N/N1003A cells homogenized in 50 mM Tris/Cl, pH 7.5, 20 mM $MgCl_2$, 0.1% protease inhibitor (Sigma), 5 mM NaF, and 2 mM PMSF. The soluble and membrane fractions were separated with centrifugation at 100,000 x g for 1 hr at 4 °C. The pellet fraction was suspended in the same buffer and both fractions were resolved with SDS PAGE and WB. C=control; H=H_2O_2 (100 μM, 20 min.)

B. Enzyme activity



PKCγ enzyme activity was measured with the PepTag assay kit. Equal protein amounts of whole cell extracts (N/N1003A) were immunoprecipitated with anti-PKCγ antisera for 4 hrs. at 4 °C. The bead complexes were recovered and incubated with the PKCγ reaction mixture. The resulting fluorescent phospho-peptides were resolved on a 0.8% agarose gel. The intensities were measured with spectrophotometry at 570 nm. C=control; H=H202 (100 μM, 20 min.) Insert: protein control.

C. PKCγ phosphorylation on Thr514

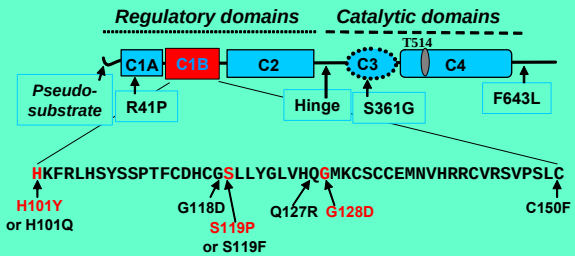


Autophosphorylation of Thr^{514} of PKCγ was determined in N/N1003A cells. The whole cell homogenates were normalized for protein concentration and resolved by SDS PAGE and WB. The blots were probed with anti-PKCγ antibodies (total PKCγ) and anti-pT514 antibodies.

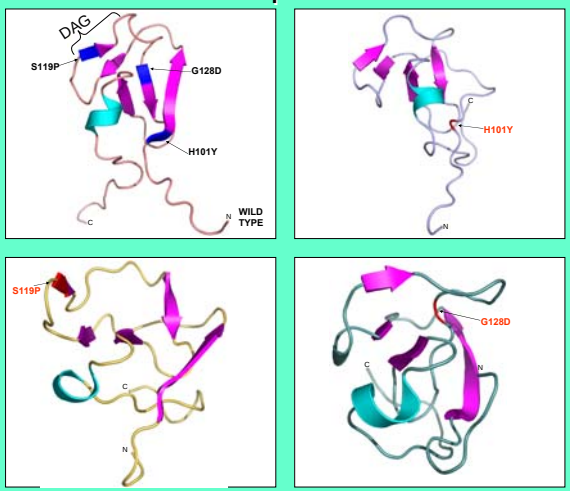
D. phosphorylation of Cx43 on Ser368 by PKCγ.

The phosphorylation state of Cx43 was determined from N/N 1003A whole cell lysate. Equal protein amounts were resolved with SDS PAGE and WB. The blots were probed with anti-CX43 or anti-pS368 antibodies.

Figure 1 Domain structure of PKCγ showing SCA-14 mutations



Molecular Computation Models



Modeling Studies

Methods: Using the computational informatics software Sybyl 7.0, three single amino acid mutate monomer processes were performed on the PDB file (1TBN) of the C1B domain of PKCγ attained from RCSB Protein Data Bank. After the mutation, a standard side chain torsion scan was administered for all rotateable side chains to minimize Van der Waals contacts. A dynamic process was applied to allow the chain to overcome low kinetic barriers and to cover a wider section of conformational space. Last a subset energy minimization was performed adjusts the geometry of the protein to find the lowest total energy.

Results: The results of our molecular modeling indicate that SCA-14 mutations within the C1B domain of PKCγ may affect the activation mechanism of PKCγ. Although many of the residues in the C1B domain participate in binding to activators and responding to oxidative stress, our results indicate that each mutation may cause specific problems for the enzyme. For example, modeling conformational changes within the C1B domain, including a collapse in the DAG binding loop, predict that the enzyme's functionality is altered by the mutations.

Conclusions: Mutations which are associated with Human Spinocerebellar Ataxia SCA-14 cause structural and biochemical changes within the C1B domain of PKCγ that affect the mechanisms by which this enzyme becomes activated. This alters the enzyme's ability to phosphorylate Cx43 and results in a failure of gap junction control.

Support:

This work was supported by EY13421(DT) & the Undergraduate Fellowship from KSU Terry C. Johnson Center for Basic Cancer Research (to D.S., D.M., & L.G.)