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Sequence Analysis and Potential Action of Eukaryotic Type Protein Kinase from *Streptomyces coelicolor* A3(2)

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Abstract

Protein kinase C (PKC) is a family of kinases involved in the transduction of cellular signals that promote lipid hydrolysis. PKC plays a pivotal role in mediating cellular responses to extracellular stimuli involved in proliferation, differentiation and apoptosis. Comparative analysis of the PKC-$\alpha, \beta, \varepsilon$ isozymes of 200 recently sequenced microbial genomes was carried out using variety of bioinformatics tools. Diversity and evolution of PKC was determined by sequence alignment. The ser/thr protein kinases of *Streptomyces coelicolor A3 (2)*, is the only bacteria to show sequence alignment score greater than 30% with all the three PKC isotypes in the sequence alignment. *S.coelicolor* is the subject of our interest because it is notable for the production of pharmaceutically useful compounds including anti-tumor agents, immunosupressants and over two-thirds of all natural antibiotics currently available. The comparative analysis of three human isotypes of PKC and Serine/ threonine protein kinase of *S.coelicolor* was carried out and possible mechanism of action of PKC was derived. Our analysis indicates that Serine/ threonine protein kinase from *S. coelicolor* can be a good candidate for potent anti-tumor agent. The presence of three representative isotypes of the PKC super family in this organism helps us to understand the mechanism of PKC from evolutionary perspective.

Keywords: eukaryotic type protein, evolution, protein kinase C, *Streptomyces coelicolor*

Introduction

Protein kinase C (PKC) a family of kinases, involved in the transduction of signals for cell proliferation and differentiation, is an 80KDa enzyme that transduces the cellular signals to promote lipid hydrolysis (Nishizuka, 1995). This is recruited to the plasma membrane by the second messenger diacylglycerol. PKC phosphorylates tyrosine residues in certain target proteins, which control growth and cellular differentiation (Filner, 1999). PKC plays a pivotal role in mediating cellular responses to extracellular stimuli involved in proliferation, differentiation, apoptosis, and exocytotic release in a number of non-neuronal systems such as islet cells, chromaffin cells and paramecium (Ohkusu *et al*, 1995). PKC has also been implicated in neoplastic transformation & carcinogenesis. Tumor cell invasion renders it a potentially suitable target for anticancer therapy (Yoshiji *et al*, 1999).

PKC isozymes contain four conserved regions termed C1-C4. C1 contains a cysteine-rich motif and forms the diacylglycerol-binding site (Newton, 1995). The auto inhibitory pseudosubstrate sequence is upstream of the cysteine-rich motif in the same region (House *et al*, 1987). C2 contains the recognition site for acidic lipids and, in some isozymes, the Ca2+ binding site. C3 and C4 form the ATP and substrate binding lobes of the kinase, respectively (Newton, 1995). In the inactive form, the pseudosubstrate domain is bound to the catalytic domain of PKC (Orr *et al*, 1994). Upon stimulation, PKC translocates to the plasma membrane where the C1 and C2 domains interact with DAG and phosphatidylserine, respectively. This interaction causes the pseudosubstrate domain to dissociate from the catalytic domain, which results in activation of PKC. Inactive PKC is not freely distributed throughout the cytoplasm but appears to be localized to specific sites within the cell. Association of PKC with scaffolding proteins such as AKAP79 (A Kinase-Anchoring Protein 79) (Klauck *et al*, 1996) and Gravin (Nauert *et al*, 1996) facilitates localization.

Streptomycetes are ubiquitous soil bacteria, and they play a key role in the global carbon cycle by degrading the insoluble remains of other organisms. More clues to the development of the PKC super family come from the study of the bacterium *Streptomyces coelicolor*. *S. coelicolor* has a large collection of enzymes and can metabolize many diverse nutrients. This extremely simple organism contains approximately 8,667,507bp, yet has complex life cycle exhibiting mycelial growth and spore formation (Bentley, 2002) and notable for production of pharmaceutically useful anti-tumor compounds. Of the predicted genes, an unprecedented proportion carries out regulatory functions in the cell (Winstead, 2002). More than twelve percent of the genome is involved in facilitating biological processes, such as the bacterium's

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response to environmental stimuli and stress. The chromosome of *S coelicolor* is long with a G+C content of 72.1% and is predicted to contain 7825 protein encoding genes. Analysis of these sequences predicts the presence of 3 PKC-related proteins, alpha, beta and epsilon in this organism. *S. coelicolor* A3 (2) is apparently being used an extremely manipulatable and powerful genetic system (Bentley *et al*, 2002). The presence of three representative isotypes of the PKC super family in this organism helps us to understand the mechanism of PKC from evolutionary perspective.

The major aim of our present work is to understand the role of PKC family in general and to use the protein kinase family as a model to look at sequence evolution in higher organisms. 200 medically significant bacterial genomes were selected and studied from the list of recently sequenced organisms. In this article, we determine and analyze the sequence homology of microbial genomes with that of human PKC's using various bioinformatics tools. Furthermore, the evolution of action of mechanism of PKC is discussed from the evolutionary perspective.

Method

Comparative analysis of the PKC-$\alpha, \beta, \varepsilon$ isozymes of 200 recently sequenced microbial genomes, that were found in ergo integrated genomics (http://ergo.integratedgenomics.com/ERGO) and NCBI (http://www.ncbi.nlm.nih.gov) websites, was carried out using variety of bioinformatics tools. Protein sequences of the three human isotypes of PKC and Serine/threonine protein kinase of *S.coelicolor* were obtained in fasta format from pfam-wellcome trust Sanger's institute. These two sequences were blasted at the same time using NCBI-blast2seq to obtain the percent identity and the alignment of sequences. The domain structure was obtained from pfam-graphical view of the domain structure for sequence comparison. Multiple sequence alignment of all the four sequences (PKC-$\alpha, \beta, \varepsilon$ & serine/ threonine protein kinase from microbe) was done using EMBL-EBI ClustalW and the conserved consensus sequences were obtained using the jal view.

Results

Comparative analysis of the $\alpha, \beta, \varepsilon$ isotypes of PKC with 200 completely sequenced genomes resulted in only two genomes with significant sequence similarity score in all the three isotypes. The ser/thr protein kinases of *S.coelicolor A3 (2)*, *Enterococcus faecium DO (JGI)* are the only bacteria to show sequence alignment score greater than 30% ,with all the three PKC isotypes, in the protein sequence alignment (Fig. 1). *S.coelicolor* is the subject of our interest in this study, because of its association with production of pharmaceutically useful anti-tumor drugs. After the sequencing of the genome in 2002, it is now clear that serine/threonine kinase represents the sole PKC in *S.coelicolor*. The protein kinase of the bacterium shows high homology with the PKCs, and its domain structure suggests similar enzymatic properties. It has a Gln/Pro rich domain that contains the invariant residues required to confer calcium binding as the C-2 domain of the PKC's and also a

PKC epsilon

	Isotype of PKC	C1-1	C1-1	C-2	PKC core	PKC terminal	Low complexity Region
1	Beta & Epsilon	65	63	No similarity	66	46	No similarity
2	Alpha & Epsilon	65	61	No similarity	66	47	No similarity
3	Alpha & Beta	92	77	71	83	80	–
4	Serine/threonine protein kinase from *Streptomyces coelicolor* (8-276 residues) & Alpha	–	–	–	33	–	–
5	Serine/threonine protein kinase from *Streptomyces coelicolor* (8-276 residues) & Beta	–	–	–	32	–	–
6	Serine/threonine protein kinase from *Streptomyces coelicolor* (8-276 residues) & Epsilon	–	–	–	28	–	–

Fig. 1. Protein domain architecture of PKC:-

well-conserved PKC domain.

It is evident from our analysis (Fig. 2) that the sequence of ser/thr protein kinase is highly conserved in the regions from 54～57 and 137～141. This highly conserved region corresponds to regions around 100 for PKC α & β and 240 region for PKC-ε that further fall in the C1 domain of PKC. This is the region where proteins bind to DAG/ PE and bring down the activation of the enzyme. This structural similarity facilitates the binding of ser/ threonine protein kinase of *S.coelicolor* to the DAG/PE binding site, there by likely inhibits the binding of PKC to the activation receptors- C-1 domain. The PKC core domain in the activated state doesn't have a binding site to bind and under goes proteolysis-causing inhibition of the entire down stream pathway. This happens due to competitive binding of ser/thr kinase resulting in inhibition of PKC. Thus the microbe's protein kinase is more than likely to function as anti-tumor drug. The protein kinase of bacterium is presumed to act by competing at PKCs conserved C-1 domain thereby inhibiting the enzyme activity irreversibly. Conserved sequences are the regions of having high catalytic activity and normally function as active sites.

Due to the structural similarity of *S.coelicolor* kinase with PKC, its kinase competitively inhibits the binding of PKC to the phorbol-ester domain thus inhibiting the for-

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A_HUMAN-------MADVFP--GNDSTASQDVANRFARKGALRQ----KNVHEVKDHKFIARFFKQP 47
B_HUMAN-------MADPAA--GPPPSEGEESTVRFARKGALRQ----KNVHEVKNHKFTARFFKQP 47
E_HUMANPEGRVYVIIDLSGSSGEAPKDNEERVFRERMRPRKRQGAVRRRVHQVNGHKFMATYLRQP 180
ser/thr----------------LLTAQIADALAASHADGVVHR----------DLKPANVLLKQT 33
                        .    :           ::          . *     ::*.

A_HUMANTFCSHCTDFIWG-FGKQGFQCQVCCFVVHKRCHEFVTFSCPGADKGPDTDDPRSK----- 101
B_HUMANTFCSHCTDFIWG-FGKQGFQCQVCCFVVHKRCHEFVTFSCPGADKGPASDDPRSK----- 101
E_HUMANTYCSHCRDFIWGVIGKQGYQCQVCTCVVHKRCHELIITKCAGLKKQETPDQVGSQRFSVN 240
ser/thrGGEMHPMLTDFG-------IARLADSPGLTRTHEFVGTPAYVAPESAEGRPQTSAVD--- 83
          *     :*        .::.     .* **::   .    :        *

A_HUMAN--HKFKIHTYGSPTFCDHCG-SLLYGLIHQGMKCDTCDMNVHKQCVINVPSLCGMDHTEK 158
B_HUMAN--HKFKIHTYSSPTFCDHCG-SLLYGLIHQGMKCDTCMMNVHKRCVMNVPSLCGTDHTER 158
E_HUMANMPHKFGIHNYKVPTFCDHCG-SLLWGLLRQGLQCKVCKMNVHRRCETNVAPNCGVDAR-- 297
ser/thr-VYGAGILLYELVTGRPPFGGGSALEVLHQHLSAEPRRPSTVPDPLWTVIERC------- 135
           :*  *   *     * .    :::* :...    ..      .*   *

A_HUMANRGRIYLKAEVADEKLHVTVRDAKNLIPMDPNGLSDPYVKLKLIPDPKNESKQKTKTIRST 218
B_HUMANRGRIYIQAHIDRDVLIVLVRDAKNLVPMDPNGLSDPYVKLKLIPDPKSESKQKTKTIKCS 218
E_HUMAN-------------------------------GIAKVLADLGVTPDKITNSGQRRKKLIAG 326
ser/thr-------------------------------------LRKNPDDRPSAENLARGLRVV 156
                                            *   **   .: :  : :

A_HUMANLNPQWNESFTFKLKPSDKDRRLSVEIWDWDRTTRNDFMGSLSFGVSELMKMPASGWYKLL 278
B_HUMANLNPEWNETFRFQLKESDKDRRLSVEIWDWDLTSRNDFMGSLSFGISELQKASVDGWFKLL 278
E_HUMANAESPQPASG---SSPSEEDRSKSAPTSPCD--------------QEIKELENNIRKALS 368
ser/thrAEGIGVHANSAQIGAAENVGALLAP--------------------DPAPAQVPGAPDAA 195
       :     :       :::     .                       :

A_HUMANNQEEGEYYNVPIPEGDEEGNMELRQKFEKAKLGP---AGNKVISPSEDRKQPSNNLDRVK 335
B_HUMANSQEEGEYFNVPVPPEGSEANEELRQKFERAKISQGTKVPEEKTTNTVSKFDNNGNRDRMK 338
E_HUMANFDNRGEEHRAASSPDG-----------------------QLMSPGENGEVRQGQAKRLG 404
ser/thrYDPNGATSVLP----------------------------HTSGPAGAADPTAVLPSTG 225
       : .*     .                              :   .

A_HUMANLTDFNFLMVLGKGSFGKVMLADRKGTEELYAIKILKKDVVIQDDDVECTMVEKRVLALLD 395
B_HUMANLTDFNFLMVLGKGSFGKVMLSERKGTDELYAVKILKKDVVIQDDDVECTMVEKRVLALPG 398
E_HUMANLDEFNFIKVLGKGSFGKVMLAELKGKDEVYAVKVLKKDVILQDDDVDCTMTEKRILALAR 464
ser/thrAPDP--TAVMPPVPPGQPGAPGQGGPEDPHPWQNQLRAARDRNEQTQVQYLDPNQDPLRR 283
         :     *:   . *:   .   * :: :. :   : .  ::::.:    : .  .*
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Fig. 2. Sequence conservation in the ser/thr protein kinase motif of *S.coelicolor.*

mation of tumors. This function enlists the microbe as an anti-tumor agent. The current knowledge of the PKC signaling pathway (activation of conventional PKC) as well the activations that are indicated from our analysis, in humans in tumor formation, is shown in Fig. 3A, B. External stimulus activates a G-Protein-Coupled Receptor (GPCR), which in turn activates a stimulating G-protein. The G-protein activates phospholipase C (PLC), which cleaves phosphoinositol-4, 5-bisphosphate (PIP2) into 1,2-diacylglycerol and inositol-1, 4,5-trisphosphate (IP3). The IP3 interacts with a calcium channel in the endoplasmic reticulum (ER), releasing Ca2+ into the cytoplasm. The increase in Ca2+ levels activates PKC, which translocates to the membrane, anchoring to diacylglycerol (DAG) and phosphatidylserine. The active PKC transduces down stream signals activating many pro-

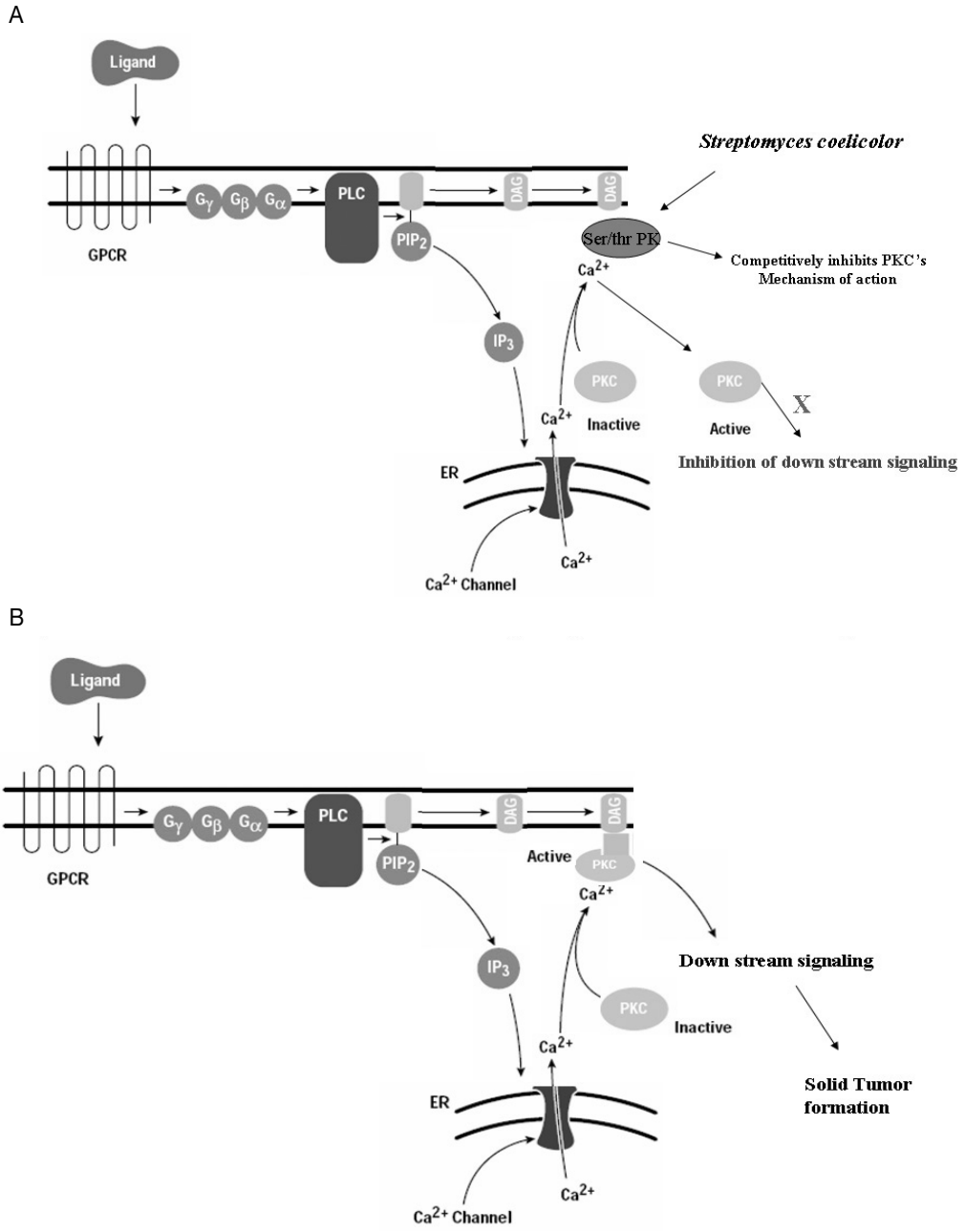

Fig. 3. (A) PKC activation and mechanism of signaling in the presence of ser/thr protein kinase. (B) PKC activation and mechanism of signaling in the absence of ser/thr protein kinase.

teins ultimately leading to the formation of tumors by activation of angiogenesis promoting protein.

Discussion

Although the structure of PKC is not known, we know for sometime that the isozymes of PKC are homologous with cAMP-dependent protein kinase (protein kinase A) (Mellor *et al*, 1998). The consensus sequence of protein kinase C enzymes is similar to that of protein kinase A, because it contains basic amino acids close to the Ser/Thr to be phosphorylated. PKC is distributed ubiquitously in variety of organisms. Biochemical, molecular cloning, and immuno-cytochemical analysis has revealed the existence of multiple subspecies of PKC in various mammalian tissues as well as in other organisms such as *Xenopus laevis* (Chen *et al*, 1989), *Dictyostelium* (Luderus *et al*, 1989; Jimenez *et al*, 1989), Sea urchin eggs (Shen *et al*, 1989), and *Drosophila* (Rosenthal *et al*, 1987; Schaeffer *et al*, 1989). An enzyme similar to PKC has been identified in *Saccharomyces cerevisiae* (Ogita *et al*, 1990) and a gene that encodes a nucleotide sequence with 50% identity to the sequence of the mammalian PKC is also been isolated. PKC is not a single enzyme but a family of kinases with at least 10 isoforms that are synthesized as single polypeptides with N-terminal regulatory domain (20～40kDa) and a C-terminal catalytic domain (～45kDa).

Examination of *S.coelicolor* protein sequence shows that the domain sequence 8-276 exhibits protein kinase activity and has ＞28% identity with the core protein kinase C activity of all the isotypes of PKC (Fig. 1). In the mammalian PKC super family, the various regulatory modules are differentially distributed between the different isotypes. However all of these elements are present together in *S.coelicolor,* suggesting that this serine/ threonine protein kinase represents an archetypal PKC. The structural similarity of ～30% between PKC isotypes and *S.coelicolor* protein kinase and the conserved sequences provides insight about the development of inhibitors of protein kinase C from *S.coelicolor.* Protein kinase C catalyzes the phosphorylation of tyrosine residues in certain proteins. A protein which stimulates angiogenesis, e.g. of solid tumors, called Vascular Endothelial Growth Factor (VEGF), depends in part on activation of a protein which is a receptor for VEGF. The activation is achieved by phosphorylation of the VEGF receptor, which in turn is catalyzed by a protein kinase C. Drugs which inhibit protein kinase C can promote death of tumor cells. Drug companies have been developing inhibitors of protein kinase C, for quite sometime now, because a number of processes important in certain diseases, notably solid tumors, are facilitated by the action of protein kinase C. Applications of the microbe as anti-tumor drug due to the structural similarity of the microbes protein kinase with C-1 domain is inevitable in this case. During the activation of PKC in the signaling pathway serine/threonine protein kinase from *S. coelicolor* competitively binds to the Diacyl glycerol/Phorbol ester-binding domain inhibiting the binding of active PKC. This will in turn devoid the PKC enzyme of its substrate further inhibiting all the down stream pathways activated by PKC. Thus serine/ threonine protein kinase acts as anti-tumor agent. The current PKC inhibitor that has been reviewed here is relatively non-specific in its action; i.e does not fully exploit the potential for differential inhibition of PKC functions or specific isoenzymes. The role of PKC in tumor formation and apoptosis suggests that combination of PKC inhibitors with conventional cytotoxics may be effective for complete treatment. The *S.coelicolor* sequence provides a new drive and dimensions for researchers trying to develop pharmaceuticals through the genetic engineering of bacteria.

Future Perspectives

The function of serine/threonine protein kinase in *S. coelicolor* is not yet known. Our analysis gives new directions to the researcher in this field of anti-tumor drugs. Eventually, the clues to its function may come from its localization & structure. The structure of PKC provides a starting point for the drug design of high potency inhibitors of the catalytic activity of PKC. Catalytic key residues (Lys-137, and Asp-141of *S.coelicolor* kinase), invariant in all protein kinases, preserve intramolecular interactions observed in active kinase structures, in accordance with the structural criteria used to define catalytically active kinase conformations. Our future work may focus on predicting consensus secondary structure for the *S.coelicolor* kinase from a combination of the multiple sequence alignments, probabilities for formation of loop, a-helix, and b sheet regions and an average hydropathy plot. Studies on the endogenous protein by Immuno techniques may bring light on the distribution of this protein kinase in the bacterium and this knowledge can eventually be utilized to understand the functionality of this protein. It may be possible to develop agents that target a single isoenzyme in the near future. Furthermore, as the downstream events resulting from PKC activation are being characterized it may be appropriate to target events further down the signaling pathways rather than concentrating on events happening in the activation process. Further research is needed to establish if inhibition of PKC isoenzymes by *S.coelicolor* protein kinase will prove beneficial in con-

trolling and terminating tumors.

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