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Research Article

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Effect of Magnesium ions on Phosphoinositide-3-Kinase C2_β C2 Domain

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ABSTRACT

Phosphoinositide-3- kinases (PI3Ks) are widely involved in cellular processes and can phosphorylate the 3-hydroxyl position of the inositol ring of phosphoinositides. Enhancement of P13Ks functions in cellular processes cannot be overlooked in protein interactions and signal trafficking. The effect of magnesium ions on phosphoinositide-3-kinase C2 β C2 domain was aimed to be investigated. The amplification of a DNA fragment of PI3K C2 β C2 domain using polymerase chain reaction (PCR) technique was employed. The oligonucleotide primers (anti sense, sense) were mixed with buffer, dNTPs, vent DNA polymerase and variable concentration (500, 1000 and 1500 μ M) of magnesium sulphate. The PCR products were analysed using agarose gel electrophoresis and purified with using QIAquick PCR purification and purification of the amplified gene product. The successful qualitative PCR analysis of human PI3K C2 β C2 domain in the absence of an additional magnesium ions yielded the specific amplicon of the desired product size below 4000bp and PCR products were successfully purified to remove debris from the products. The purified PCR products can be used for cloning.

Keywords: Magnesium ions, Phosphoinositide-3-Kinase (PI3K), PI3K C2β C2 domain, PCR technique

INTRODUCTION

A family of enzymes known as Phosphoinositide-3-kinase (PI3K) consists of three classes (class I, class II and class III) which are major parts of signalling pathways [14], that involve in various cellular activities [4,11]. PI3K characterization were based on structural features and substrates specificity [4, 19]. Foster et al. [10] reported that the class II PI3K has three known isoforms (PI3K C2 α , PI3K C2 β and PI3KC2 γ). It has been reported that this class II P13K helps in stem cell factor and epidermal growth factor dependent signals [3], but may not appear to be directly activated by Ras [11]. Moreover, the most relevant isoform (PI3K C2β) is made up of a PI kinase domain, a Ras-binding domain, catalytic domain and C2 domains at both C and N termini, but lack regulatory subunits. as shown in Figure 1 as reported by researchers[1,10,12]. The C2 domain at the C terminus is about 126 amino acid residues (1484-1609) and \sim 378 base pairs. This particular domain may be involved in Ca²⁺ dependent or Ca²⁺ independent phospholipid membrane binding. If it is involved in a Ca²⁺ dependent manner, the Ca²⁺ binding site will likely involve residues in more than two loops at a particular end of the domain most times termed as the calcium binding regions (CBRs). Arcaro et al. [1] reported that PI3K C2β C2 domain may bind Ca²⁺ with low affinity when compared to PI3K C2 α domain of synaptotagmin and the absence of C2 domain in PI3K C2 β could result in raising the activity of lipid kinase. This is because PI3K C2B contains substrate specificity such as phosphatidylinositol (PtdIns) and the complementary DNA (cDNA) of human PI3K C2_β with a C2 domain (one of the conserved regions) which was successfully cloned and the enzyme was expressed in mammalian cells. Some characteristics of PI3K C2β can be viewed in Table 1. Some domains found in PI3K C2β mediate interactions between proteins and bind to membrane lipids had been reported by [7]. The conserved domains present in most of these proteins were involved in enzymatic activity, protein interactions, DNA binding as well as in other crucial cellular processes [15]. PI3K C2 β has been said to be important in cell migration in some epithelial lines [14] and it is also in the differentiation of HL-60 hematopoietic cells by retinoic acid [20].



Figure 1: The structural features of PI3K C2β consisting of C2 domain, a helical domain, ras-binding domain and a catalytic domain. PI3K C2β lacks regulatory subunits but have N and C terminal extensions attached to the PI3K core structure, which could mediate protein-protein interactions [19]

Fable 1: Characteristics of PI3	K C2β adapted from [18	1
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Catalytic subunit	Binding site	Regulated by	Distribution	In vitro Lipid products	Functions for selective inhibitors
C2β	Clathrin	RTK, GPCR, IR	Widespread	PI(3)P PI(3,4) P2	Vesicle trafficking, cell migration, liver growth and liver regeneration.

Domin et al. (1997) have carried out studies on most of the C2 domains in PI3K_68D, m-cpk, PKC, PI3K C2 α and PI3K C2 β and it seems C2 domain in particular cellular protein plays a significant role in membrane trafficking or signal transduction but there is limited report on the effectiveness of PI3K C2 β C2 domain. In this study, the effect of magnesium (Mg²⁺) concentration on amplified gene products of PI3K C2 β C2 domain was aimed to be investigated.

EXPERIMENTAL SECTION

2.1 Materials used for PCR

The template DNA (PI3K C2 β C2 domain) and oligonucleotide primers as presented in Table 2 were obtained from Biomedical Laboratory, University of Bedfordshire labelled with JD 88, JD 89, JD 90 and JD 91, Vent DNA polymerase (0.04 units/µl) (each forward {sense} primer was used in conjunction with the reverse {antisense} primer) (1µM), ThermoPol Reaction buffer, dNTPs (200µM), magnesium sulphate (MgSO₄) and autoclaved Milli-Q- purified water. Other materials used were a bucket half filled with ice, Gibson pipette, Eppendorf well plate, micro test tube, agarose gel, centrifuge and Gel Doc-It^{Ts2} Imager.

Тε	ıble	2:	An	antisense	primer	and	three	sense	primers	used	in	PC	R

Primers	Sequence
JD 88	Antisense- 5'CGGAATTCTTATTACAAGGTGCCATGACTTCGAGA 3'
JD 89	Sense - 5'GCCCCGGGAAATAAACTCTTCATCATGGTG 3'
JD 90	Sense - 5'GCCCCGGGATCCTACAAAAACAATAAACTC 3'
JD 91	Sense - 5'GCCCCGGGAAAGCTGTCTATCTCCTACAAA 3'

A, C, G and T represent nucleotide of adenine, cytosine, guanine and thymine at a position, respectively.

2.2 Amplification of target gene using PCR

Vent DNA polymerase and dNTPs stocks were placed on ice. One PCR-ready Master Mix plate and the block containing the two sets of primers to be used in the reactions were thawed and centrifuged using Jouan Centrifuge at speed of 1000 rpm for 20 sec. The first master mix comprising of buffer, dNTPs, vent DNA polymerase, JD89, JD88 and water was prepared for 5 reactions and aliquot was divided into 4 different labelled micro test tubes of A1, A2, A3 and ANC. The next master mix comprising of buffer, dNTPs, vent DNA polymerase, JD90, JD88 and water was prepared for 5 reactions and aliquot was divided into 4 different labelled micro test tubes of B1, B2, B3 and BNC. The last master mix comprising of buffer, dNTPs, vent DNA polymerase, JD91, JD88 and water was prepared for 5 reactions and aliquot was divided into 4 different labelled micro test tubes of B1, B2, B3 and BNC. The last master mix comprising of buffer, dNTPs, vent DNA polymerase, JD91, JD88 and water was prepared for 5 reactions and aliquot was centrifuged at speed of 1000 rpm for 20 sec. Afterwards, MgSO₄ in varied concentration of 500µM, 1000µM and 1500µM, respectively, was added to the respective tubes and template DNA was added with the use of a Gibson pipette, to most of the micro test tubes. The Eppendorf well plate was centrifuged again at the same speed, then and placed in the Thermo cycler (BIO-RAD Laboratories Inc., California, USA) for the purpose of optimization of the initial PCR with magnesium (Mg²⁺) of different concentrations.

The cycling conditions applied were as follow:-1.95° C for 2 mins (denaturation) 2.95° C for 1 min (primer annealing) 3.55° C for 1 min (extension) 4.72° C for 1 min (final extension) 5. Repeat steps 2-4 for 34 times 6.68° C for 5 min

7.4°C hold, infinity

Afterwards, the amplified products were analysed on 1% Agarose gel. The product on the gel was viewed using the Gel Doc-It^{Ts2} Imager.

Reaction components	A1	A2	A3	ANC	B1	B2	B3	BNC	C1	C2	C3	CNC
ThermoPol reaction buffer (µL)	5	5	5	5	5	5	5	5	5	5	5	5
F. Primer (µL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
R Primer (µL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DNTPs (µL)	1	1	1	1	1	1	1	1	1	1	1	1
Water (µL)	41	40	39	39	41	40	39	39	41	40	39	39
Vent DNA (µL)	1	1	1	1	1	1	1	1	1	1	1	1
$MgSO_4(\mu M)$	-	500	1000	1500	-	500	1000	1500	-	500	1000	1500
Template DNA (µL)	1	1	1	-	1	1	1	-	1	1	1	-
Total (µL)	50	50	50	50	50	50	50	50	50	50	50	50

Table 3: Composition for the PCR reaction

A1, B1 and C1 are samples without MgSO₄; A2, B2 and C2 have 1µl of MgSO₄ in their reactions; A3, B3 and C3 have 2µl of MgSO₄ and ANC, BNC and CNC are negative control samples containing JD89+88, JD90+88 and JD91+88 primers, respectively, as well as 3µl of MgSO₄ in their reactions.

2.3 Purification of PCR products after amplification.

The PCR products (A1, B4 and C7) were purified using QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA). Following the manufacturer's protocol, the Buffer PB containing guanine chloride, denaturant and protein detergent and the PCR product obtained were mixed, placed into a QIAquick column, and centrifuged for 30-60s at 13,200 rpm to bind DNA. The Buffer PE was put into the QIAquick column and centrifuged at 13,200 rpm for 30-60s to elute DNA. Then, flow through the QIAquick column and placed in a fresh tube. The products were centrifuged at 13,200 rpm for 1min and eluted with autoclaved Milli- Q- purified water. The purified PCR products were analysed on 1% Agarose gel.

RESULTS AND DISCUSSION

The PCR result obtained from gel electrophoresis can be seen in Figure 2 and clearly stated in Table 4. In a nut shell, DNA bands were present in all reactions without $MgSO_4$ and in some reactions with $1\mu M$ of $MgSO_4$. Lane 1, with a label SM (selectable marker), has bands of 1kb (Kilo Base) molecular weight marker. All DNA bands were seen below 400bp when viewed along with the selectable marker. PCR products were separated by 1% Agarose gel electrophoresis compared with 1Kb molecular weight marker to see the size of base pairs and the concentration of magnesium required.



Figure 2: Initial PCR for optimization

Reaction in each lane Primers	Master mix+ template DNA - MgSO ₄ (Lane 2, 6 and 10)	Master mix+ template DNA+ 1µl MgSO ₄ (Lane 3,7 and 11)	Master mix+ template DNA+ 2µl MgSO ₄ (Lane 4,8 and 12)	Master mix+ 3µl MgSO ₄ - template DNA (negative control) (Lane 5,9 and 13)
A (JD89+88)	Thick DNA bands present	Faint bands present	No DNA band	No DNA band
B (JD90+88)	Thick DNA bands present	Thick DNA bands present	No DNA band	No DNA band
C (JD91+88)	Thick DNA bands present	Thick DNA bands present	Faint bands present	No DNA band

Table 4: Summary of the initial PCR for optimization

Based on agarose gel electrophoresis analysis, non-purified and purified amplified PCR products were shown in in Figure 3(a) and (b), respectively. The DNA bands of the amplified PCR products obtained after using the primers (A, B and C) and without the addition of MgSO₄ were observed below 400bp. Although, the thickness of the DNA bands was more in the non-purified than the purified PCR products. In the purified PCR products, the DNA band in B4 containing primers (JD 90+88) was quite fainter than the DNA bands in A1 (JD 89+88) and C7 (91+88).



Figure 3: PCR amplified products without MgSO₄(a) before purification (b) after purification

The detection of the presence of PI3K C2 β C2 Domain and determination of its DNA size and yield was successfully carried out by experimental PCR and gel electrophoresis, respectively. The total base pairs expected was about 378 and Figure 3(a) indicated that the size of the DNA was expected to be relative the same size to the marker (between 350bp and 400bp) and also, there was absence of DNA bands in the control wells. This, however, proofs qualitatively that the PCR products yielded the PI3K C2 β C2 Domain. The expected total number of the amplified products was 17,179,869,184 copies after 34 cycles. The performance of PCR was effective, efficient and enhanced by the primer design, annealing temperature, reaction buffer, vent DNA polymerase DNA quantity and DNA quality. This is in agreement with the report of [2].

However, 1.0 μ M concentration of each primer was sufficiently used during the reaction. The optimal primer annealing temperature was dependent on the base composition (i.e., the proportion of A, T, G, and C nucleotides), primer concentration, and ionic reaction environment. The annealing temperature and the components of the ThermoPol Reaction Buffer especially the cations were also considered after designing the primers for proper annealing. The special cation combinations were present to maintain high primer annealing specificity and majorly, the free Mg²⁺ ion present in the reaction buffer intention was to make vent DNA polymerase more active. Vent DNA polymerase was used rather than *Taq* polymerase to revert the error generated due to exonuclease activity which can affect the sequence of protein [17] and it has been proven to be much better than *Taq* polymerase in terms of fidelity [9, 13] because of its heat resistance, ability to generate new DNA strands using the DNA template and primers during elongation.

During the experiment, magnesium sulphate was added to the 2^{nd} , 3^{rd} and negative control samples of A, B and C to determine the effect of its concentration in the reaction. The magnesium ion (Mg²⁺) was expected to act as a vital Vent DNA polymerase cofactor necessary for enzyme activity as well as binding to DNA, primers, and nucleotides contained in the amplification reaction. After the successful PCR was carried out, it can be observed from Figure 2

that the products show the variation in DNA band thickness due to the presence of the Mg^{2+} in the reaction because the wells which contained all the necessary reagents and materials without magnesium had thick DNA bands and some other wells (B1 and C1) with 1µM of magnesium sulphate had thick bands too. This may have occurred because the free magnesium already present in the reaction buffer was sufficient enough to generate efficient products when bound to the DNA template, primers and nucleotides during amplification. Moreover, it can also be observed that the addition of 500 μ M Mg²⁺ into sense primer of JD 89, JD 90 and JD 91 incorporated with antisense JD88 increased the thickness of band but otherwise when the concentration of Mg²⁺ increased to 1000 μ M and 1500 μ M Mg²⁺. This implies that the need to minimize the concentration of Mg²⁺ in the reaction is necessary for higher fidelity of amplified products because excessive magnesium stabilized the double stranded DNA, thus denatured the DNA which reduced the PCR product yield. This is in agreement with the report of [16]. This is to say that reduction in DNA band may be attributed to change in sense primer sequence. This indicated that reaction exist between the sense primers and the Mg^{2+} and optimal products can be obtained at concentration of Mg^{2+} in buffer solution and when added with $500\mu M Mg^{2+}$. The free magnesium in the buffer was also determined by the proportions of the DNA template and primer used for this PCR optimization [6]. Therefore, running the experimental PCR reactions with the concentration of magnesium as the only variable and also running the reaction on the agarose gel proved the most effective magnesium concentration by the presence of most DNA bands (PCR products).

The essence of purifying the DNA was to clean it up and remove debris from single copy genes in genomic DNA or pathogenic viral DNA sequences in genomic DNA isolated from an infected organism in the products. It was observed that the concentration of the purified DNA products in all wells were different. The second purified DNA product had the lowest concentration whereas the first purified DNA had the highest. This could be as a result of reagents used. The purified products are now prepared for DNA sequencing and restriction enzyme digestion.

CONCLUSION

The required components for PCR such as DNA template, DNA polymerase (Vent), primers (three sense primers and one antisense primer), buffer solution, deoxynucleoside triphosphates (dNTPs), magnesium ion and also, the PCR thermal profile were used according on a small scale to detect the presence of Human PI3K C2 β C2 domain DNA, determine the effect of magnesium concentration on amplification and purify the amplified DNA product. The qualitative PCR analysis of Human PI3K C2 β C2 domain in the absence of an additional Magnesium yielded clear DNA bands of the amplified products below 400bp and the amplified products were successfully purified to remove debris from the products. This preliminary step for molecular DNA cloning was successful. For further research, this experiment can be carried out on a large scale to generate larger amount of purified products that will be applied in Restriction Enzyme Digestion and ligation with a plasmid vector to generate a recombinant DNA molecule, which could be further used to generate a fusion protein. The fusion protein will further be used to detect the binding partners of PI3K C2 β C2 domain thereby determining the function of the PI3K C2 β enzyme.

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