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PH DOMAIN OF G PROTEIN-COUPLED RECEPTOR KINASE-2 BINDS TO PROTEIN KINASE C (PKC) AND NEGATIVELY REGULATES ACTIVITY OF PKC KINASE

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1. ABSTRACT

G protein-coupled receptor kinase-2 (GRK),also known as beta1-adrenergic receptor kinase(beta-ARK1), plays an important role in agonist-induced desensitization of the beta-adrenergic receptors. Activation of protein kinase C (PKC) is able to stimulate phosphorylation and activation of GRKs and induce desensitization of G protein-coupled receptor. However, detail mechanism of interaction between PKC and GRK_2 and the effect of GRK_2 on activity of PKC remain unknown. Pleckstrin homology (PH) domain is a kind of functionally domain containing about 120 amino acids, which exists on many protein molecules that involve in cellular signal transduction. A PH domain located in GRK2 residue 548 to 660 may play a significant role in mediating interaction between PKC and GRK2. In present study, we revealed that PKC could associate with PH domain of GRK2 in pull-down assay *in vitro*. Co-immunoprecipitation displayed binding of PKC to GRK2 in intact Jurkat cells after prolonged stimulation of epinephrine. Assay of PKC beta1 kinase activity indicated that the binding of the PH domain of GRK2 to PKCbeta 1 could down-regulate activity of PKC beta 1 kinase. Thus, GRK2 may play a negative feedback regulatory role on PKCbeta1 activity in interaction between GRK2 and PKCbeta 1.

2. INTRODUCTION

G protein-coupled receptor kinase-2 is a member of the GRK family of at least 6 GRKs(1). GRKs were implicated in Agonist-induced phosphorylation and homologous desensitization of G protein-coupled receptor (GPR). Phosphorylation sites of the GPR by GRK were located in the carboxyl tail of the receptor and involved amino acid residues are at positions serine 404,408,and 410(2). These phosphorylation sites are thought to initiate desensitization of GPR.

It has been recently reported that protein kinase C (PKC) could phosphorylate and activate GRK which sequentially mediated desensitization of GPR(3). In addition, PKC could directly phosphorylate GPR and initiate desensitization of the receptors(4). Thus, PKC likely participates in phosphorylation and heterologous desensitization of adrenergic receptor in multiple levels. Furthermore, both activated GRK and PKC are recruited to the specific membrane and GRK may be localized to the membrane via its PH domain binding to phosphatidylinositol phosphates. Both recruitments to membrane will facilitate the association of PKC with GRK. Deletion or mutation of the PH domain will abolish GRK faculty of phosphorylating and activating GPR (5). PH domain of GRK plays a pivotal role in mediating the phosphorylation and desensitization of the receptors. However, detail mechanism of PKC how to interact with and activate GRK has been unclear.

PH domain has been distinguished from more than 100 molecules that were implicated in signaling and other biological function. The main function of PH domains is to mediate protein-phospholipid and protein-protein interaction. Up to now, several molecules such as phosphatidylinositol phosphates, G beta gamma, G alpha

subunit-12, RACK1, PKC and F-actin have been identified as ligands of PH domains (6-11). It was reported by Yao *et al* (1994) that the PH domain of Btk and Itk interacted with protein kinase C (10). Afterward, the interactions between PKC and other PH domains were also confirmed by different groups. Those PH domains that could associate with PKC share a high homology in 1^{st}-4^{th} beta strands(12)which formats a relatively conserved face binding to PKC. But other PH domains that can interact with PKC remain to be identified.

PKC is a family of serine/threonine protein kinase, which play significant roles in numerous cellular responses (including cell proliferation, differentiation, growth control, tumor progression, apoptosis and etc.)(13). Besides the event of PKC phosphorylating GRK2 discussed above, PKC can phosphorylate GRK5 and modulate the activity of GRK5(14); PKC can also lead to olfactory signal termination and desensitization via other GRKs(15); Agonist stimulation results in oxytocin receptor interaction with GRK and PKC (16). Moreover, PKC also presented a manner of phosphorylating directly muscarinic receptor and leading the receptor desensitization (17).

Like most interaction between signaling molecules, PKC likely associates with GRK2 via some motif(s) before phosphorylating and activating GRK2. To elucidate mechanism of the details of interaction between GRKs and PKC, we report the study on interaction between PKC and GRK2. It is expected that present study provide new insights for understanding of the modulation mechanism of PKC in GRKs mediating signaling pathway. It is also expected that present investigation can illustrate the importance of the GRK2 PH domain in interaction with PKC.

3. MATERIALS AND METHODS

3.1. Materials

Anti-GRK2, anti-PKCβeta1 and secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology, PKC assay kit (Signa TECT™ PKC assay system) and reverse transcription kit were from Promega, glutathione-agarose beads was from Sigma, expression vector pGEX-4T-1 and ECL detection kit(for western blotting) were from Amersham Pharmacia Biotech., PVDF membrane was from Millipore, rabbit IgG was from Sino-American company (China). Protein A-agarose was from Pierce Inc.

3.2. Construction of expression vector pGEX-PH

The mRNA of human GRK2 (beta1-adrenergic receptor kinase) was isolated from human fresh placenta(18), and reverse transcription was performed according to the kit protocol. cDNA of PH domain(residue 548 to 660) (19) was amplified by PCR with oligonucleotides containing Bam HI sites (forward, CGGGATCCGGCCACGAGGAAGACTAC) and Eco RI sites (reverse, GGAATTCTCACCGCTGCACCAGCTGCTG). The products were purified from agarose gel and the resulting fragments were digested with Bam HI and Eco RI, respectively. The resulting fragments were ligated in frame into between Bam HI and Eco RI sites of plasmid pUC-19. The resulting plasmid(termed pUC-PH) was resolved by sequencing. The fragments were excised from pUC-PH and ligated in frame into expression vector pGEX-4T-1, named pGEX-PH.

3.3. Expression and purification of GST fusion protein

GST-GRK2 PH domain, Btk PH domian and GST were expressed in *Escherichia coli* strain DH5. Here was Btk PH domian positive control(10). The expression was induced with 0.2 mM of IPTG at 26 ? for 1 hour in LB medium containing 1% glucose. Briefly, the bacteria were resuspended in ice-cold STE buffer (10 mM Tris-HCl, 150 mM NaCl,1.0 mM EDTA,1.0 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml lysozyme, pH 8.0) on ice for 20 min. The treated bacteria were added dithiothreitol to 5.0 mM and sarkosyl to 1.5% on ice until the solution become viscous, and then lysed by sonicate (30-40 output,1 min ×2,at 4 ? C). Cell lysate was centrifuged at 10,000×g for 20 min at 4 ? C. Component of the both supernatant and pellet were separate by SDS-PAGE to determine distribution of the fusion protein. The supernatant was added Triton X-100 to 2%, and then filtered with 0.45 μm membrane. The solution was save as the fraction containing interested proteins, and then treated glutathione-agarose beads was added to solution at 4 ? C for 2 hours with occasional gentle mixing. The beads were washed with PBS and centrifuged at 800× g,4 ? C for 3 min×5 times. The pellet was subjected to SDS-GAGE in 12% gels. In order to utilize equivalent amount of GST and fusion proteins for the following assay, protein concentration was modulated according to band sizes in SDS-GAGE gel.

3.4. Determination of GRK2 expression in mammalian cell lines

In order to confirm a cell line expressing both GRK2 and PKC beta1, MDCK, SGC-7901, DU145,PC-3, U937 and Jurkat cells were maintained in Dulbecco's modified eagle's medium or 1640 medium with 10%(v/v) new born bovine serum and a 5% CO_2 humidified atmosphere at 37? C. In brief, the 10^7 cells were harvested by centrifuging or scraping off, respectively, washed by PBS at 4 ? C and then incubated with 1 ml of lysis buffer (20 mM Tris-HCl,pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 1 mu g/ml aprotinin) on ice for 20 min with casually and vigorously vortexing . The mixtures were centrifuged at 12,000× g for 10 min at 4 ? C. The supernatant s were saved as GRK2-contained preparations for western blotting.

3.5. Cell culture and lysis

Jurkat T lymphocytes were maintained as above with 1640 medium. The 2×10^7 cells were divided 2 parts and were serum-starved for 8 h. followed by 0.5 h treatment without (Control) or with epinephrine. The stimulation was stopped by removal of the medium and addition of 3 ml of ice-cold phosphate-buffered saline, which was also used for two similar washing steps with centrifugation at 500 ×g for 5 min.

3.6. Protein-protein interaction assay *in vitro*

Approximately 20 μg of the GST and GST fusion

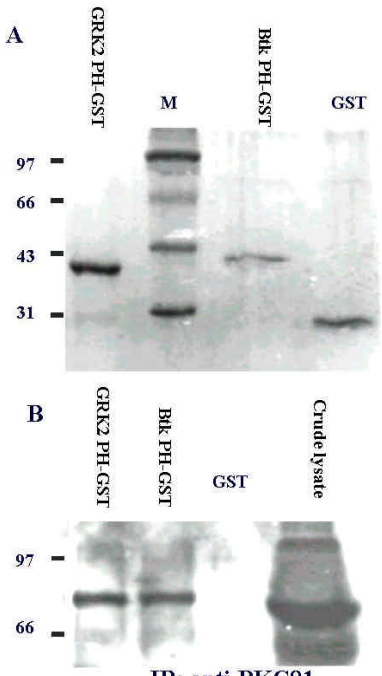

Figure 1. Binding of purified GST-GRK2 PH domain and GST-Btk PH domain with PKC. (*a*) Expressed GST-GRK2 PH domain, GST-Btk PH domain fusion protein and GST were purified, as described above, analyzed by SDS/PAGE, and visualized by staining with Coomassie blue. Molecular mass markers shown are 97.4, 66.2, 43, 31, 21 and 14.4 kDa. (*b*) Immunoblotting with anti-PKC beta1 antibody was used to assay binding of PKC to GST-GRK2 PH domain, GST-Btk PH domain fusion protein and GST which previously bound to glutathione-agarose. Jurkat cell crude lysate (containing PKC beta 1 protein) was incubated with equivalent amounts of GST-GRK2 PH domain, GST-Btk PH domain fusion protein or GST alone. After washing for a few times, precipitated proteins were analyzed by immunoblotting with antibody against PKCβ1, and visualized as described in Materials and Methods.

proteins immobilized on glutathione-agarose beads was incubated with the 0.3 ml of Jurkat T lymphocyte supernatant at room temperature for 2 hours with gentle mixing. The beads were then washed five times with 1 ml of ice-cold PBST(PBS with 0.05% tween-20). The eventual bead pellet was resolved by SDS-PAGE. Equivalent amount of the fusion protein or GST alone were used for following assay.

3.7. Western blotting

The resolved proteins were electrotransferred onto PVDF membrane. Membrane was blocked by incubating with 5% gelatin in PBST at 37 °C for 1 h with gentle vibration, and then incubated with 1 mu g of anti-PKC beta1 antibody at 4 ? C overnight. The membrane was then washed 5 times with PBST and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody at room temperature for 1 h with gentle vibration. Membrane was finally washed 6 times with PBST to remove unbound secondary antibody and visualized by using ECL (Amersham Pharmacia Biotech).

3.8. Co-immunoprecipitation assays

10^7 Jurkat cells were lysed in 1 ml of lysis buffer(20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.02% sodium azide, 0.5 mM phenylmethylsulfonyl fluoride, 2 mu g/ml leupeptin and 2 mu g/ml aprotinin). Briefly, 10 μg of anti-PKC beta1, or anti-GRK2 antibodies and rabbit IgG were added to 200 μl of the lysate, respectively, and then samples were incubated overnight at 4? C. The products were added 40 mu l of immobilized protein A(Pierce Inc) and incubated at room temperature for 2 h with gentle mixing. Products were added 0.5 ml lysis buffer (without protease inhibitor) and centrifuged at at 500 ×g,4 ? C for 3 min. The supernatant was Discarded and this procedure was repeat for 6 times. In brief, the pellets were subjected to SDS-PAGE. The proteins were electrotransferred into PVDF membranes. The membranes were blocked with gelatin and incubated with anti-PKC beta1 or anti-GRK2 antibodies, respectively. Membranes were probed and visualized as described above.

3.9. PKC Kinase Assay

The fusion proteins and GST were purified as described above ,and washed extensively with PBST. After washing, the GST-fusion proteins and GST were eluted with 20 mM of reduced glutathione in 50 mM Tris·HCl (pH 8.0). The elution proteins were concentrated using a Centricon (Millipore). Protein concentrations were determined with Bradford reagent (Bio-Rad). Equivalent amounts of the protein were added to the 100 mu l lysate of Jurkat cells. According to manufacturer's instruction (Signa TECT™ PKC assay system), activity of PKC in the lysate was determined by subtracting the radioactivity of the reaction with control buffer and 100 muM PKC peptide inhibitor from that of the reaction without activation buffer neither PKC inhibitor. The experiment was performed in triplicate samples for each group. Data are presented as percentage of PKC activity and control was used as 100%.

4. RESULTS

4.1. Binding of GRK2 PH domain to PKC *in vitro*

To search for potential binding between the expressed proteins (GST- GRK2 PH domain, GST-Btk PH domain and GST) and PKC beta1, the proteins immobilized on glutathione-agarose beads were with lysate of Jurkat cells, and then the products were subjected to SDS-PAGE and western blotting. The potential PKCbeta 1 was probed with anti- PKC beta 1 antibody. Binding of Btk PH domain to PKC beta 1 was formerly approved(10).Here the Btk PH domain served as positive control and GST protein was used as negative control. As shown in Figure 1, GST fusion proteins encompassing GRK2 PH domain and GST-Btk PH domain associated specifically with PKC beta 1, but the GST did not pull down PKC beta 1 under the same experimental conditions. We confirmed the binding of GRK2 PH domain to PKC beta 1 was specific *in vitro*.

4.2. Binding of GRK2 to PKC upon prolonged stimulation of epinephrine

In order to detect association of GRK2 with PKC

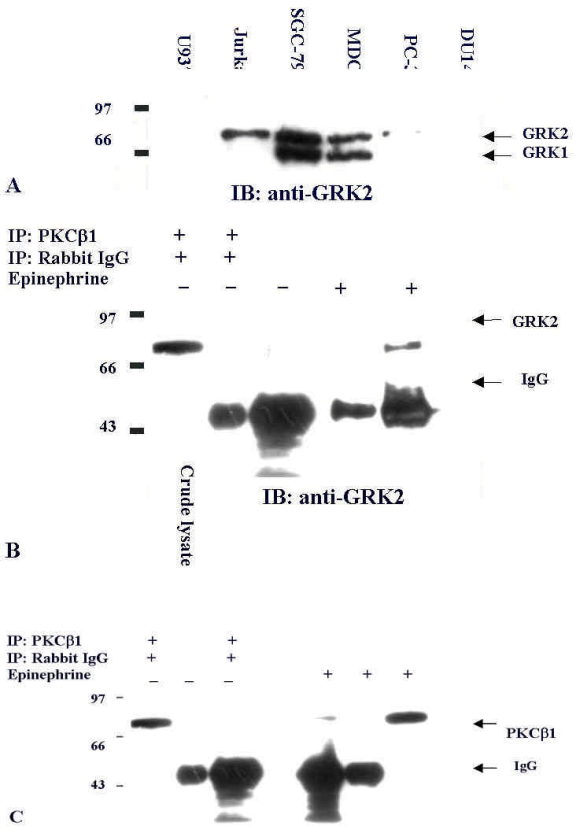

Figure 2. Epinephrine causes binding of PKC to GRK2 in Jurkat cells. (A)Western blots reveal the presence of GRK2 proteins in mammalian cell line. MDCK, SGC-7901, DU145, PC-3, U937 and Jurkat cells were lysed and equivalent amounts of the proteins (20 μg) were fractionated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the anti-GRK2 antibody (diluted 1:1000) as described above. The PVDF membrane was visualized by using ECL. PKC keeps dissociation from GRK2 in intact cells. Serum-starved intact jurkat cells were stimulated with 5 mmol/L epinephrine for 30 minutes or without stimulation of epinephrine. Totale cell lysate was prepared for co-immunoprecipitation with anti-PKC beta 1,anti-GRK2 anti-bodies, or rabbit IgG as negative control. The cell extracts were separated by SDS-PAGE, transferred to PVDF membranes. Anti-PKC beta 1,anti-GRK2 anti-bodies were used to detect presence of GRK2 and PKC beta 1, respectively. The membranes were finally visualized using ECL as described above in the experimental procedures. (B) The precipitated proteins with anti- PKC beta 1 and IgG were detected by anti-GRK2 anti-body. Serum-starved intact jurkat cells (without stimulation of epinephrine) were shown in lane 1,2 and 3, and cells with stimulation of epinephrine were shown in lane 5 and 6. Crude lysate served as positive control for GRK2 protein. (C) The precipitated proteins with anti-GRK2 and IgG were detected by anti- PKC beta 1 anti-body. The cells were treated as in (B) and Crude lysate served as positive control for PKC beta 1 protein. One representative of three experiments is shown.

in vivo, expression of GRK2 of 6 cell lines were confirmed by western blotting in advance. The same amount of the protein was utilized in SDS-PAGE. The results indicated GRK2 were expressed in PC-3, MDCK, SGC-7901 and Jurkat cell lines. Because of cross-reactive with GRK1, the GRK2-antibody detected out GRK1 in MDCK and SGC-7901 cell lines, but the antibody can specifically recognize GRK2 in Jurkat cells. Thus, the following Co-immunoprecipitation assays was performed with Jurkat cells in which PKC beta 1 was also expressed.

Phosphorylation and activation of GRK by PKC enhances heterologous desensitization of G protein-coupled receptor. In the pull-down assay, present results indicated that PH domain of GRK2 associated specifically with PKC *in vitro*. To determine the association of PKC with GRK2 *in vivo* after stimulation of agonist of beta-AR, 100 μm epinephrine was added to the cells in 1640 medium for 30 min. Co-immunoprecipitation was performed with specific anti-GRK2 and anti-PKC antibodies in co-precipitation to detect PKC and GRK. After stimulation of 100 μm epinephrine for 30 min, PKC was detected out in precipitate of GRK antibody, and GRK was also detected out in precipitate of PKC antibody. In contrast, the PKC did not bind to GRK2 in the intact cells without stimulation of epinephrine. Thus, results indicated that the association of PKC with GRK2 was involved in GRK2 activation and desensitization of G protein-coupled receptor.

4.3. Effects of GRK2 and Btk PH domains on PKC activity

Effects of proteins on PKC activity were determined as described under Experimental Procedures. After purification procedure as described above, the expressed proteins were eluted and resolved in PBS. Equivalent amounts (20 μg) of GST- GRK2 PH domain, GST-Btk PH domain and GST were added to the lysate of Jurkat cell lysate (100 μl) in determining PKC activity. Yao *et al* reported that Btk associated with PKC, Btk activity was down-regulated by PKC. In present study (Figure 3), GRK2 PH domain associated with PKC and down-regulated PKC activity but Btk PH domain up-regulated PKC activity compared with GST and crude lysate (control).

5. DISCUSSION

The GRK family is composed of six members, named GRK 1-6. Of these, GRK2 phosphorylates G protein-coupled receptors (GPCRs) and plays an important role in mediating homologous desensitization of GPCRs. Chuang's work shown the activity of GRK2 was increased in a protein kinase C (PKC)-dependent manner(20). Further study demonstrated that PKC could directly phosphorylate GRK. This study suggests that GRK can be activated via phosphorylation by PKC. Recent work has led to the identification of phosphorylation site at GRK2, the study of Krasel and co-worker show PKC phosphorylated GRK2 at serine 29 (21), but detail mechanism of PKC beta 1 recognizing and binding to GRK2 remains unknown. In present study, we report that GRK2 can bind to PKC via its

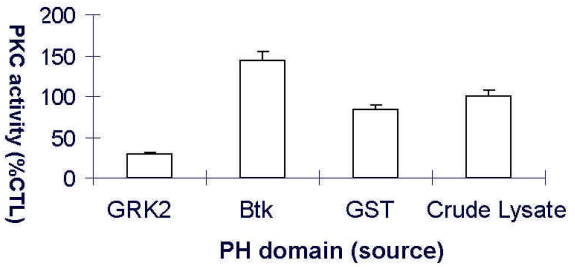

Figure 3. Effects of GRK2 and Btk PH domain on PKC activity in Jurkat cells. Jurkat cells were serum-starved for 8 h and then the cell lysate was prepared. 20 μg of GST and fusion proteins was added to 100 μl of the cell lysate (containing intact PKC kinase activity) and PKC activity was determined as described under experimental procedures. The data were normalized as the percentage of the PKC activity with control as 100%. The data are the means ± S.E. of triplicate for each treatment group.

Table 1 First three beta sheets of PH domains of GRK2 and GRK3 are compared with other PH domain known or predicted to bind to PKC

PH domain (source)	β1	β2	β3
Btk	**ESIFLKT**---SQQKKKTSPLS--N**FKKRLFLLT**-VH--**KLSYY**EYDF		
β-spectrin	**MEGFLNRKH**-EWEAHNKKASSR-S**WHNVYCVIN**-NQ--**EMGFY**KDAK		
dynamin	**RKGWLTI**---NNIGIMKGGS-----**KEYWFVLT**--AE--**NLSWY**KDDE		
AFAP,Nt PH domain	**ICAFLLRKK**-RFGQ----------**WTKLLCVIK**-EN--**KLLCY**KSSK		
Consensus	1* +*	++*****	+L 1p
GRK2	**MHGYMSKM**--GNPFLTQ-----WQ**RRYFYLF**---PN--**RLEWR**GEGEA		
GRK3	**MHGYMLKL**--GNPFLTQ-----WQ**RRYFYLF**---PN--**RLEWR**GEGES		

Consensus sequence is shown and symbols used are as in the paper of Baisden *et al.*:1 indicates aromatic residues, + indicates positively charged residues, * indicates hydrophobic residues, p indicates polar residues. The sequences of GRK2 and GRK3 were from Fushman paper(23). The previous study revealed PKC inhibited the activity of Btk kinase via binding to Btk PH domain (10), but the binding effect on PKC activity remains unclear. In present study, the effects on PKC activity of Btk and GRK2 PH domains , as well as GST(a control) were determined. Compared with GST or control, Btk PH domain could increase PKC kinase activity, but GRK2 PH domain inhibit PKC kinase activity. The mechanism of activation or inhibition of PKC activity may lay in the GRK2 PH domains binding to PKC regulatory domain and regulating activity of PKC kinase. Biological function of Btk PH domain inhibiting PKC activity will be laid in future investigation. Present study indicated that PKC through its binding to the PH domain of GRK and facilitates phosphorylation and activation of GRK by PKC under stimulation of epinephrine. On the other hand, GRK2 PH domain appears to play a negative feedback regulatory role on PKC activity. GRK2 can immediately turn off PKC kinase activity on GRK after PKC completes phosphorylation and activation of GRK.

carboxyl-terminal PH domain *in vitro*, which is a fundamental event in mediating interaction between PKC and GRK2. The present result suggests the GRK PH domain has likely an analogous fashion to PH domain of Btk and Itk, Which could directly interact with protein kinase C in the previous study(10). The PH domain of GRK2 is probably involved in directly binding of GRK2 to PKC and facilitates phosphorylation and activation of GRK2 by PKC.

Stimulation of agonist (epinephrine) on adrenergic receptor results in association of PKC beta 1 with GRK2 in Jurkat cells. GRK2 could through its PH domain directly bind to PKC and followed phosphorylation by PKC. But in intact cells (without stimulation of epinephrine), PKC and GRK2 keep in dissociation state. The epinephrine stimulation on adrenergic receptor may activate phospholipase C (PLC), and initiates a cascade of events. The stimulation causes PLC-mediated hydrolysis of inositol phospholipids, and result in calcium mobilization and production of second messenger diacylglycerol (DAG). The DAG and Ca^{++} are activators of PKC, and cause activation of PKC (22). In present study, activated PKC is likely to directly bind to and phosphorylate GRK2, which phosphorylates G-protein coupled receptor and results in receptor desensitization. In this signaling pathway, activation of PKC appears to be prior to receptor desensitization and is of fundamental importance in regulating the procedure of receptor desensitization.

Those PH domains from Btk, beta-spectrin, dynamin and amino-terminal of AFAP-110 known directly binding to PKC share a high homology at first three beta-sheets (Baisden JM *et al.*,2001)(12). These beta-sheets form conserved structure elements and may specifically bind to PKC. Table 1 shows the residue comparison of PH domains from GRK2 and GRK3(23) with above PH domains. The PH domain of GRK2 and GRK3 match that consensus well in first 1-3 beta-sheets which may serve as a structure base of PH domains binding to PKC. Thus, the PH domain of GRK2, as well as GRK3 has potential to binding to PKC.

6. ACKNOWLEDGEMENT

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Key Words: G-Protein-Coupled Receptor Kinase-2, Protein Kinase C, Pleckstrin Homology Domain, Binding

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