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Phosphorylation of Tyrosine 256 Facilitates Nuclear Import of Atypical Protein Kinase C

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Abstract Herein, we employed a combined approach of molecular modeling and site-directed mutagenesis to address the role of tyrosine phosphorylation in transport of atypical protein kinase C (aPKC) into the nucleus. Computer modeling of the three-dimensional structure of the aPKC catalytic core, reveals that tyrosine 256 (Tyr256) is located at the lip of the activation loop and is conserved among members of the aPKC family, ι/λ and ζ. Based on these findings, we examined whether tyrosine phosphorylation of aPKC on the activation lip may facilitate nuclear import. An antiserum was generated that selectively recognizes the phosphorylated Tyr256 residue in aPKC. By isolating nuclei of PC12 cells and immunoprecipitating aPKC with Ab-PY256, we observed that Tyr256 is rapidly phosphorylated upon NGF treatment prior to entry of aPKC into the nucleus. aPKC was observed to exclusively bind to importin-β. The interaction between importin-β and aPKC was enhanced upon tyrosine phosphorylation of aPKC and binding was abrogated when Tyr256 was mutated to phenylalanine. We propose that phosphorylation of aPKC at Tyr256 induces a conformation, whereby, the arginine-rich NLS is exposed, which then binds importin-β leading to import of aPKC into the nucleus. Altogether, these findings document a novel role for the tyrosine phosphorylation in regulating import of atypical PKC into the nucleus. J. Cell. Biochem. 85: 42–53, 2002.

Key words: phosphorylation; Src; atypical PKC; PC12 cells; nuclear import

The atypical protein kinase Cs (aPKCs) are a serine/threonine kinase comprised of PKC-ζ, PKC-ι isoforms and its mouse homolog PKC-λ that are 97% homologous [Zhou et al., 1994, 1997], and are regulated by mechanisms other than diacylglycerol (DAG), Ca^{2+}, or phorbol esters [Kochs et al., 1993]. Phosphoinositol-3-kinase (PI3K) and its lipid product, phosphotidylinositol-3,4,5-triphosphate (PIP_3), serve as a second messenger to activate the aPKCs [Nakanishi et al., 1993]. Previous studies have shown that the aPKCs increase in the nucleus of nerve growth factor (NGF)-stimulated PC12 cells [Wooten et al., 1997; Zhou et al., 1997] and play a role in regulating both differentiation and survival signaling [Coleman and Wooten, 1994; Wooten et al., 2000]. NGF stimulation also results in an increase in nuclear PI3 kinase, leading to an increase in PIP_3 that precedes activation of PKC-ζ in the nucleus in PC12 cells [Neri et al., 1999]. Upon entry into the nucleus, aPKCs locate to the inner nuclear matrix where they bind to chromatin [Wooten et al., 1997] and perform critical roles in regulation of transcription, ribosomal RNA biosynthesis and shuttling of mRNAs by phosphorylating various nuclear targets such as: SP1 [Pal et al., 1998], NF-κB [Martin et al., 2001], nucleolin [Zhou et al., 1997], and hnRNP A1 [Municio et al., 1995].

Proteins that translocate to the nucleus must pass through the nuclear pore complex (NPC), a gateway through the nuclear envelope. Passage through the NPC is highly regulated by receptor-transporters that selectively bind short regions of amino acids termed the nuclear localization sequence (NLS) [Jans et al., 2000]. A structural change induced by phosphorylation may result in exposure of an NLS sequence allowing binding to receptor complexes followed by subsequent transport into the nucleus. Two "classical" basic NLSs that have been described are a lysine-rich amino acid sequence observed in T antigen and a bipartite amino acid sequence of two basic regions separated by a spacer region [Jans et al., 2000]. The importin-β

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receptor can independently regulate the import of proteins containing basic arginine residues that resemble the importin-β binding (IBB) domain of importin-α [Palmeri and Malim, 1999]. Phosphorylation may also enhance recognition of a protein by importins [Hubner et al., 1997] by inducing a conformation that exposes the NLS, thereby increasing the affinity for binding to importin-β leading to nuclear import [Xiao et al., 2000].

Several studies have clearly documented aPKC nuclear translocation and localization [Wooten et al., 1997; Zhou et al., 1997; Neri et al., 1999; Perander et al., 2001]. However, little is known about the structural mechanisms utilized in transport of aPKC into the nucleus. In vivo studies in PC12 cells revealed that aPKC exists as a substrate of the tyrosine kinase Src [Seibenhener et al., 1999]. The specific tyrosine residues in aPKC phosphorylated by Src have been identified [Wooten et al., 2001]. Modeling studies depicted herein reveals one of these tyrosine residues, Tyr256, resides at the lip of the activation loop of aPKC and may thus play a role in import. In support of this idea, conformational change of protein structure induced by a negative charge of a phosphate group in the lip of the activation loop occurs in both ERK2 and MAPKK, where nuclear translocation depends upon the phosphorylation state at these specific residues [Khokhlatchev et al., 1998; Tolwinski et al., 1999].

Atypical PKCs possess an arginine-rich bipartite motif [Wooten et al., 1997], which serves as a functional NLS [Perander et al., 2001] and is conserved among the aPKCs isoforms [Perander et al., 2001]. The presence of a bipartite motif and a site for tyrosine phosphorylation at the lip of the activation loop suggests that entry of aPKC into the nucleus might be regulated by tyrosine phosphorylation at this site. We demonstrate that tyrosine phosphorylation plays a novel role in regulating entry of aPKC into the nucleus by promoting interaction with importin-β. NGF-induced src phosphorylation of aPKC exposes the otherwise hidden NLS, which then binds directly to importin-β and triggers entry of aPKC into the nucleus.

MATERIALS AND METHODS

Materials

PC12 cells, HEK 293, and SF-9 cells were obtained from the American Type Culture Collection (Rockville, MD). The antiserum to Tyr256-PKC-ι was produced by Alpha Diagnostic Intl. Inc. (San Antonio, TX); peptides synthesized for antibody production were provided by Mike Russ (Macromolecular Structure Analysis Facility, University of Kentucky, KY). GST-importin-α/β constructs were provided by (Dr. Harvey F. Lodish, MIT, MA). His-tagged IBB and anti-importin-β antibody were provided by Dr. Dick Gorlich (University of Heidelberg, Germany). Leptomycin B (LMB) was provided by Dr. Eisuke Nishida (Kyoto University, Japan). Anti-Src, agarose-coupled 4G10 (anti-phosphotyrosine) antibody and purified Src enzyme were obtained from Upstate Biotechnology (Lake Placid, NY). The monoclonal antibodies, anti-PKC-ι, and anti-phosphotyrosine PY20 were obtained from Transduction Laboratories (Lexington, KY.) The polyclonal anti-HA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse and goat anti-rabbit HRP conjugated secondary antibody, ECL reagents, Hyperfilm, and [γ-^{32}P]-ATP (3,000 Ci/mmol) were obtained from Amersham Pharmacia Biotech, Inc. (Piscataway,NJ). SDS–PAGE molecular weight marker was obtained from BioRad (Hercules, CA). Agarose-coupled secondary antibodies and all other reagents were obtained from Sigma (St. Louis, MO).

Computer Modeling of the Catalytic Domain of aPKCs

Modeling of the carboxy-terminal catalytic domain tyrosine phosphorylation sites of aPKCs were performed based on the crystal structure of cAMP-dependent protein kinase [Zheng et al., 1993; Taylor and Radzio-Andzelm, 1994] using SWISS-MODEL (Swiss Institute of Bioinformatics). The resulting theoretical structure was then analyzed using PROTEIN EXPLORER (Eric Martz, University of Massachusetts, 2000) and the three-dimensional model spanning amino acids 216–563 of PKC-ι and amino acids 246–569 of PKC-ζ was generated.

Cell Culture and Treatment

Rat tail collagen coated 100-mm plates were used to seed PC12 cells. The cells were grown in RPMI with 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin), and maintained in a 92% air, 8% CO_2

atmosphere as previously described [Wooten et al., 1997]. HEK 293 cells were grown in DMEM with 10% heat-inactivated fetal calf serum in a 95% air, 5% CO_2 atmosphere.

Nuclear Isolation

PC12 cell nuclei were isolated employing a previously established protocol [Wooten et al., 1997; Zhou et al., 1997]. In brief, PC12 cells were treated with NGF (50 ng/ml) for the indicated periods of time. The cells were harvested and washed with ice-cold Phosphate Buffered Saline (PBS). The cells were then placed into 500 μl Swelling Buffer (30 mM Tris-HCl pH 7.5, 1 mM β-mercaptoethanol (BME), 5 mM EGTA, 5 mM $Mg(Ac)_2$, 25 μg/ml Leupeptin, 2 μM PMSF, 0.15 μg /ml Aprotinin, 10 mM KCl) and allowed to sit for 10 min, followed by homogenization for 50 strokes with a size B pestle. The cells were centrifuged at 610*g* for 5 min and the supernatant saved and labeled as cytoplasmic fraction. The pellet was resuspended in Swelling Buffer and layered on top of an equal volume of overlay (Swelling Buffer plus 25% glycerol) and centrifuged at 610*g* for 5 min. A second overlay was added with 0.1% TX-100 and centrifuged at 610*g* for 5 min discarding the supernatant. Sonication Buffer (30 mM Tris pH 8, 100 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM BME) was added to the nuclear pellet and sonicated for 8 s. DNase (250 U/ml)/RNase (0.1 mg/ml) were added for 15 min at 4°C. Protein was then quantitated using the Bradford assay.

Immunoprecipitation

Cells were harvested and washed with PBS followed by addition of Lysis Buffer (~500 μl buffer per 100 mm plate of cells), and then transferred to microcentrifuge tubes. The cells were sonicated for 5 s and kept on ice, followed by rotation for 30 min at 4°C to release membrane-bound proteins. At this point agarose coupled secondary antibody (rabbit anti-mouse IgG) was added and rotated for 30–45 min to pre-clear samples. Samples were spun for 3 min in 4°C to remove debris and pellet the beads. A Bradford assay was performed on the supernatant to determine protein concentration. Typically, an immunoprecipitation was conducted on 500 μg protein in 1 ml of total volume. Immunoprecipitations using anti-4G10 and anti-PKC antibodies were conducted at a final concentration of 2 μg antibody/400 μg protein cell lysate. The agarose-coupled primary antibodies were rotated for 4 h at 4°C and centrifuged for 1 min to pellet beads with the bound proteins. The supernatant was then removed and the beads washed five times in 500 μl with Wash Buffer (30 mM Tris-HCl, pH 8.8, 1 mM BME, 5 mM EGTA, 5 mM $Mg(Ac)_2$, 125 mM NaCl). Finally, 1 × SB was added to beads followed by boiling for 2 min and separated on a 10% SDS–PAGE gel.

SDS–PAGE and Western Blotting

Samples were separated on a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane overnight at 75 mAmps. The nitrocellulose was then stained with Ponceau S (0.5 g Ponceau Stain dissolved in 100 ml of 5% TCA) and washed in TBS buffer (0.10 M Tris, pH 7.5, 0.1% Tween20, 137 mM NaCl) at room temperature (RT) to remove the Ponceau stain. The blot was placed in Blocking solution (7% milk (w/v) in TBS buffer) on a rocking platform for 3 h at 4°C. The Blocking solution was then removed by washing twice in TBS buffer. The blot was placed in primary antibody (PKC-ι; 1:1,000, Src; 1:1,000, 4G10; 1:1,000, PY256; 1:1,000) and allowed to incubate overnight at 4°C followed by washing for 30 min in TBS buffer at 37°C. HRP-conjugated secondary Ab (1:3,000) in TBS buffer was added for 2 h. Secondary antibody was removed and the blot was washed in TBS buffer for 1 h with changes every 15 min. The blot was incubated in ECL for 1 min and exposed to X-ray film.

Antibody Production and Screening

A peptide containing residues 252–260 was synthesized that had the Tyr256 residue phosphorylated. The PY256 peptide was conjugated to an N-terminal cysteine to keyhole limpet hemocyanin and injected into rabbits with Titermax adjuvant. Every 21 days, the sera was collected and screened for immunospecificity. Various amounts of both phosporylated and non-phosphorylated peptides conjugated to ovalbumin were separated by SDS–PAGE and used to screen the antisera by Western blotting. Intensity of immunoreactivity between sera and peptide was determined by autoradiography.

Purification of Recombinant PKC-ι

SF-9 (*Spodoptera frugiperda*) cells were seeded on 100-mm plates in IPL-41 insect medium for 1 h at 27°C. The medium was

removed, and recombinant baculovirus encoding PKC-ι was added to the cell at a multiplicity of infection = 10 plaque forming U/cell. The cells were harvested after 4 days and lysed at 4°C in PKC buffer (20 mM Tris, pH 7.5, 50 mM BME, 2 mM EDTA, 100 μM PMSF, 1% Nonidet P-40), and PKC purified as previously described [Zhou et al., 1997].

In Vitro Phosphorylation of PKC-ι

Phosphorylation of PKC-ι was performed using purified Src (3 Units) and purified PKC-ι from SF9 cells. In vitro assays were conducted with the indicated amounts of PKC-ι (0–5 μg), Src (3 U), 5 × Src Assay Buffer (100 mM Tris, 125 mM $MgCl_2$, 50 mM $MnCl_2$, 2 mM DTT, 0.25 mM $NaVO_3$), and cold ATP in a 50 μl reaction volume. The assays were initiated by adding cold ATP to a final concentration of 40 μM in a 30°C water bath for 10 min. The reaction was then stopped by the addition of 1 × sample buffer. The samples were boiled and separated by 10% SDS–PAGE.

Expression of GST-Importin-α, GST-Importin-β, and His-Tagged IBB

Bacteria transformed with either GST-tagged importin-α or importin-β were purified employing glutathione agarose where as His-tagged IBB was purified employing ProBond Resin as previously described [Xiao et al., 2000]. The purity of the protein preparations were confirmed by separating the samples on SDS–PAGE and Coomassie blue staining.

GST Pull-Down Assay

HEK 293 cells were transfected by calcium-phosphate with pcDNA-HA-PKC-ι or pcDNA-HA-PKC-ι^{Y256F} in the presence or absence of Src [Wooten et al., 2001]. The cells were harvested and lysed in GST Lysis buffer (50 mM Tris, 150 μM NaCl, 1% NP40, 50 μM NaF, 50 mM glycerolphosphate, 2 mM EDTA, 10% Glycerol). The lysates were allowed to rotate for 30 min and a Bradford Assay then determined the protein concentration. GST-importin-α or -β (10 μg) was added to equal concentrations of lysates (250 μg) and allowed to rotate for 3 h at 4°C. The GST beads were pelleted, and the supernatant was removed. The beads were washed extensively in GST Lysis Buffer plus 200 mM NaCl. Sample buffer (1 ×) was added to the beads followed by SDS–PAGE. The interaction of GST-importin-α or -β with HA-tagged PKC-ι was examined by Western blotting employing anti-HA. As control, the expression of the tagged constructs was verified by SDS–PAGE and Western blotted with anti-HA.

Immunofluorescence Microscopy

PC12 cells were grown on glass coverslips treated with polylysine in 24-well plates until confluency and transfected with HA-tagged PKC-ι or HA-tagged mutant PKC-ι Y256F employing Lipofectamine 2000 [Wooten et al., 2000]. Forty eight hours post transfection the cells were treated with LMB for 1 h, washed with PBS, and fixed with 4% paraformaldehyde for 15 min at RT, and rinsed again with PBS. Cells were permeabilized with 80% MeOH at −20°C for 10 min then washed with PBS + 0.05% Triton X-100 and rinsed again with PBS. Aldehydes were quenched with 0.05 mM NH_4Cl for 1 min and then rinsed again with PBS followed by blocking with 1% BSA/PBS for 2 h at RT. Cells were incubated with anti-HA (1:250, made in 1% BSA/PBS block) overnight at 4°C in humidified chambers and then washed. 2 × with PBS. Texas Red (8 μg/ml), was added in block for 2 h at RT in the dark. Coverslips were washed 5 × in PBS + 0.2% Tween 20, rinsed in PBS, dH_2O, blotted dry and mounted and sealed onto slides.

RESULTS

Modeling of the Catalytic Core

We have recently mapped the sites of tyrosine phosphorylation within the aPKCs [Wooten et al., 2001]. To gain insight into the possible function of the various residues, we undertook modeling studies of the catalytic core, based upon the known crystal structure of the catalytic subunit of cAMP-dependent protein kinase [Zheng et al., 1993]. Table I depicts critical conserved residues in the catalytic core of aPKC based on the crystal structure of cAMP-dependent protein kinase. Sequence comparisons between the crystal structure of PKA, and the predicted structures of Cdk2, MAPK, and aPKC were conducted to validate our predicted model based on the critical residues that are conserved within the catalytic core of the kinase family. The substitution of glycine to tyrosine in aPKC compared to PKA, Cdk2, and MAPK suggests that this tyrosine residue may be critical to the function of the aPKCs. Our model reveals that residue Tyr256 in PKC-ι and Tyr263 in PKC-ζ

TABLE I. Critical Residues Found in the Catalytic Core of Protein Kinases Based Upon Crystal Structure of cAMP-Dependent Protein Kinase

PKA	Cdk2	MAPK	PKC-ι	PKC-ζ	Function in PKA
Gly50	Gly11	Gly30	Gly252	Gly259	Loop that anchors β-PO_4
Gly52	Gly13	Gly32	Gly254	Gly261	Loop that anchors β-PO_4
Gly55	Gly16	Gly35	*Tyr256*	*Tyr263*	Loop that anchors β-PO_4
Val57	Val18	Val73	Val259	Val266	Lines adenine binding pocket
Lys72	Lys33	Lys52	Lys274	Lys281	Forms ion pair with α and β-PO_4
Glu91	Glu51	Glu69	Glu293	Glu300	Forms ion pair with Lys72
Asp166	Asp127	Asp147	Asp369	Asp376	Catalytic base
Lys168	Lys129	Lys149	Lys371	Lys378	Interacts with γ-PO_4
Asn171	Asn132	Asn152	Asn374	Asn381	Chelates inhibitory Mg^{+2}
Asn184	Asp145	Asp165	Asp387	Asp394	Chelates inhibitory Mg^{+2}
Glu208	Glu172	Glu195	Glu414	Glu421	Forms ion pair with Arg280
Asp220	Asp185	Asp208	Asp426	Asp433	Stabilizes catalytic loop
Arg280	Arg274	Arg299	Arg514	Arg519	Forms ion pair with Glu208

is located in the lip of the activation loop (Fig. 1). The orientation of Tyr256/Tyr263 at this site makes it accessible to kinases for phosphorylation.

Sequence comparisons revealed that Src-phosphorylated Tyr256 was unique to aPKC-ι, ζ, whereas, representative members of the other PKC isoforms, PKC-α and PKC-δ lack these residues (Table II). This finding further suggests a unique role for this residue in regulating the aPKCs. Since phosphorylation at the lip of the activation loop of other proteins has previously been shown to regulate entry into the nucleus and enhanced binding to importins [Khokhlatchev et al., 1998; Tolwinski et al., 1999], we hypothesized that once phosphorylated, Tyr256 may induce a conformational change leading to exposure of the otherwise buried NLS and therefore play a role in regulating entry of aPKC-ι into the nucleus. To test this hypothesis, we sought to generate antiserum specific for Tyr256.

Design and Characterization of Ab-PY256

Phosphorylated Tyr256 peptide was chosen as the phosphospecific epitope for antibody generation (Fig. 2A). Tyr 256 is highly conserved among members of the aPKC family (ι/λ, and ζ). Peptides were synthesized with an N-terminal cysteine followed by coupling to keyhole limpet hemocyanin and injected into rabbits to elicit an immune response producing

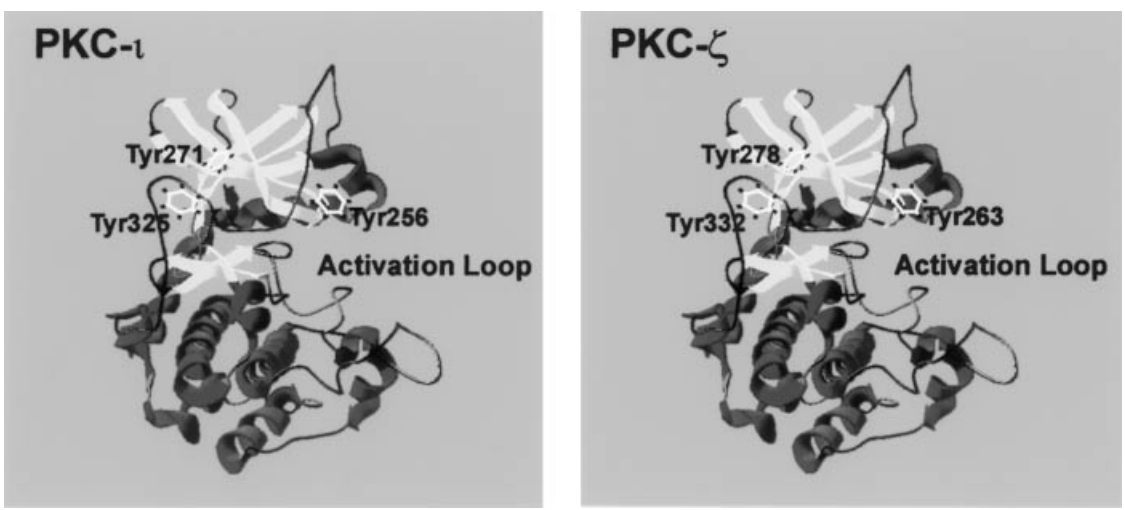

Fig. 1. Predicted carboxy-terminal structure of PKC-ι with location of tyrosine residues. Three-dimensional structure containing amino acids 216–563 of aPKC-ι and amino acids 223–570 of aPKC-ζ was determined using Protein Explorer. The location of Src-phosphorylated tyrosine residues 256, 271, and 325 [Wooten et al., 2001] are included as is the location of the activation loop. From this model, Tyr256 proximity within the lip of the activation loop can be observed.

TABLE II. Tyrosine Residue 256/263 is Novel to the aPKCs. Src-Phosphorylated Tyrosine Residues 256/263, 271/278, and 325/332 were Compared Between the Atypical (PKC-ι/ζ), Classical (PKC-α), and Novel PKCs (PKC-δ)

Y	ι/ζ	α	δ
256/263	+	−	−
271/278	+	−	−
325/332	+	+	−

(+) and (−) represents the ability of Src to phosphorylate the indicated residues.

Ab-PY256. Ovalbumin-conjugated phosphorylated and non-phosphorylated peptides were used to determine the specificity of Ab-PY256. Western blotting with Ab-PY256 against various concentrations of conjugated peptide indicates that Ab-PY256 selectively recognizes phosphorylated Tyr256 peptide rather than nonphosphorylated 256 peptide (Fig. 2B). In addition, as a negative control we synthesized a peptide, which possessed a tyrosine phosphorylated 325 residue (amino acids: FVIE$\underline{Y}^{325}$ VNGG), this peptide was also conjugated to ovalbumin and increasing concentrations were subjected to Western blot analysis employing Ab-PY256. No immunoreactivity between the peptide containing phospho-325 and the Ab-PY256 was observed (Fig. 2B). Thus, this finding demonstrates that Ab-PY256 recognizes a specific tyrosine phosphorylated site and not another tyrosine residue phosphorylated by src [Wooten et al., 2001].

Ab-PY256 was then tested to examine whether it also recognized the tyrosine phosphorylated holoenzyme of aPKC. An in vitro assay was performed, whereby, purified Src was employed to tyrosine phosphorylate aPKC-ι (Fig. 2C). Equal amounts of src (Fig. 2C, iv) and increasing amount of purified PKC-ι (Fig. 2C, iii) were incubated with cold ATP (Fig. 2C, i) or [γ-P^{32}]-ATP (Fig. 2C, ii). Ab-PY256 was employed to Western blot, the src tyrosine phosphorylated PKC-ι (Fig. 2C, i). An increase in [γ-P^{32}]-ATP phosphorylation of PKC-ι correlated with an increase in Ab-PY256 antibody recognition of tyrosine phosphorylated holoenzyme PKC-ι by Src (Fig. 2C, ii).

Since Src phosphorylated Tyr256 in aPKC is recognized by Ab-PY256 in vitro, studies were conducted to further test the specificity of Ab-PY256 in vivo. PC12 cells were stimulated with NGF, which has been shown to increase the tyrosine phosphorylation of aPKC [Seibenhener et al., 1999; Wooten et al., 2001], followed by immunoprecipitation with either Ab-PY256 or anti-G410 phosphotyrosine coupled to agarose and Western blotted with PKC-ι (Fig. 2D). These data reveal an increase in nuclear Tyr256-PKC-ι that is paralleled by an increase in the total phosphotyrosine content of PKC-ι. Overall, these data indicate that Ab-PY256 is capable of recognizing the phosphorylation state of Tyr256 in vivo and provides a tool for studying the effects of phosphorylation on the localization of aPKC-ι.

Increase in Tyrosine Phosphorylated aPKC in the Nucleus

Previous studies have shown that atypical PKCs increase in the nucleus upon NGF stimulation of PC12 cells [Wooten et al., 1997; Zhou et al., 1997; Neri et al., 1999; Perander et al., 2001]. Moreover, our model predicts that Tyr256 is located in the lip of the activation loop (Fig. 1) and location of a similar phosphorylated residue has been shown to play a role in localization of kinases like MKK1, to the nucleus [Tolwinski et al., 1999]. Therefore, studies were undertaken to determine if Tyr256 is involved in the localization of aPKC to the nucleus.

Lysates were prepared from NGF-stimulated PC12 cells and immunoprecipitated using Ab-PY256. Following NGF stimulation, phosphorylation of Tyr256 increases within 1 min and diminishes by 5–10 min (Fig. 3A). In comparison, nuclear lysates Western blotted with PKC-ι (Fig. 3A) reveal that aPKCs maximum nuclear accumulation occurs at 5 min post-NGF stimulation and declines by 10–15 min. Cytoplasmic and nuclear lysates (Fig. 3B) were probed for tubulin, an exclusively cytosolic protein, as a control to determine the purity of the nuclei. Little or no tubulin immunoreactivity could be detected in the nuclear lysates, even upon prolonged exposure of the blot. Collectively, these data reveal that Tyr256 is rapidly phosphorylated upon NGF stimulation and its phosphorylation took place prior to accumulation of aPKC in the nucleus. Moreover, pretreatment of PC12 cells with LMB, an inhibitor of CRM1-export [Fukuda et al., 1997], resulted in an accumulation of Tyr256 phosphorylated PKC-ι within the nucleus (data not shown).

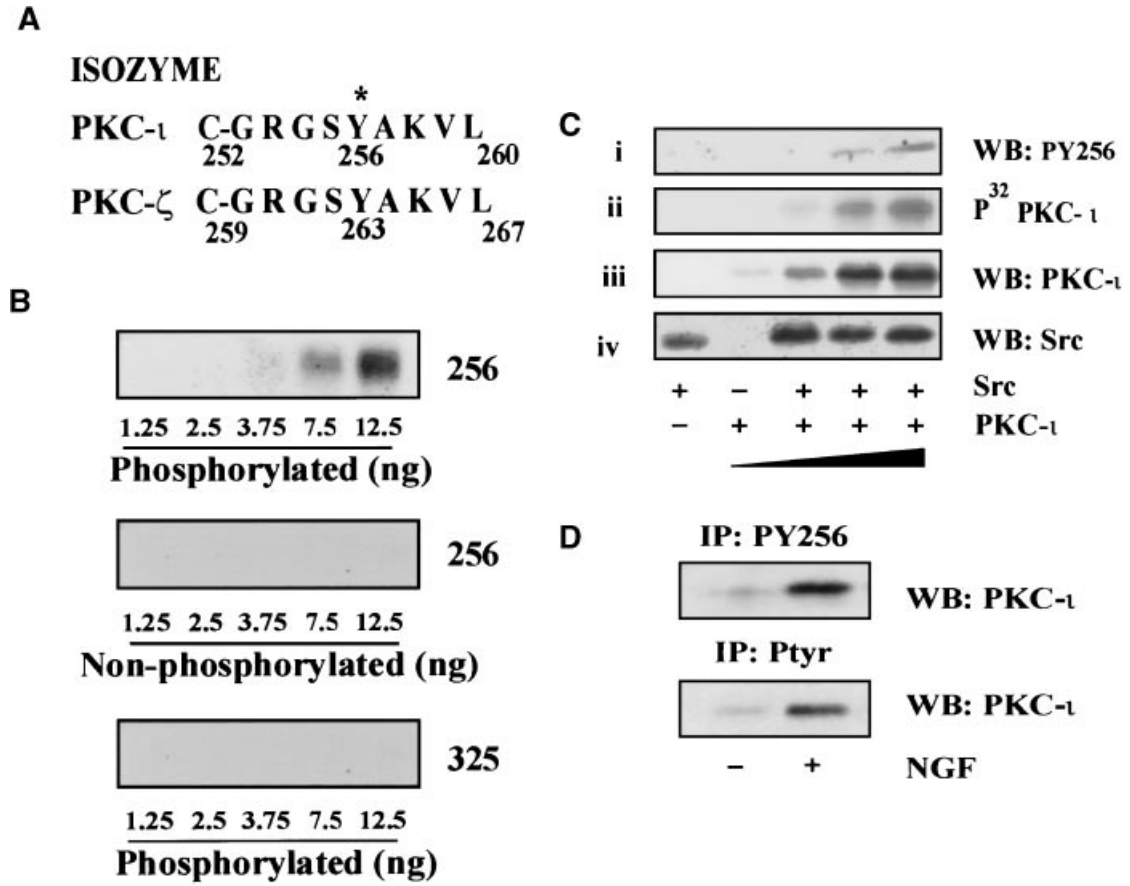

Fig. 2. Characterization of phospho-specific antibody to src phosphorylated tyrosine 256 (Ptyr256) in PKC-ι. **A**: Diagram representing the peptide used to obtain phospho-specific antibody. The two sequences indicate the amino acids surrounding Tyr256 used to construct phosphorylated peptide (* above Y indicates residue phosphorylated). The similarity of amino acid sequence between of PKC-ι and PKC-ζ are conserved between residues 252–260. **B**: Phosphorylated Tyr256 peptide was injected into rabbits to elicit an immune response producing anti-PY256 that was collected in the serum. Ovalbumin-conjugated phosphorylated (256) and non-phosphorylated (256) peptides were separated by SDS–PAGE at the indicated concentrations (1.25–12.5 ng) and a Western blot was performed using anti-PY256 antisera. In addition, as negative control the ability of anti-PY256 antisera to detect ovalbumin-conjugated phosphorylated 325 peptide was also examined. **C**: Src phosphorylates PKC in vitro and is recognized by antibody PY256. **i**: A cold in vitro kinase assay was performed by adding ATP, Src, and PKC-ι and allowed to react for 10 min at 37°C, followed by SDS–PAGE/Western blot with YP256 antisera. **ii**: As control, a reaction of this assay was performed with [γ-^{32}P]-ATP, demonstrating Src-induced phosphorylation of aPKC-ι. **iii**: Control Western blot of purified aPKC-ι included in the assay. **iv**: Western blot of purified Src included in the assay. The in vitro kinase assay was conducted +/− increasing Src concentrations and/or PKC-ι as indicated. **D**: Nuclear fraction isolated from PC12 cell lysates treated with +/−NGF were immunoprecipitated with anti-PY256 (40 μl antibody/500 μg protein) or anti-G410 ptyr (20 μl 50% 4G10-agarose/500 μg protein) and Western blotted for PKC-ι.

Co-Association of Importin-β and aPKC is Dependent on Tyrosine Phosphorylation

A mechanism proteins use to enter the nucleus is through binding nucleocytoplasmic transport proteins that can shuttle between the nucleus and the cytoplasm [Cyert, 2001]. The importin family of nucleocytoplasmic transport proteins forms a complex, made up of importin-α and -β, where importin-α contains the NLS-binding site and importin-β is responsible for docking of the importin-substrate complex to the cytoplasmic side of the NPC and its subsequent translocation through the pore [Rout and Aitchison, 2001]. Recent studies have indicated that proteins can bind exclusively to importin-β, and function independently of importin-α [Palmeri and Malim, 1999; Xiao et al., 2000; Rout and Aitchison, 2001]. Proteins that function in this manner possess an arginine-rich NLS that enables direct binding to importin-β [Palmeri and Malim, 1999]. aPKC also possesses an arginine-rich NLS (Zinc finger residues 130–152) [Perander et al., 2001],

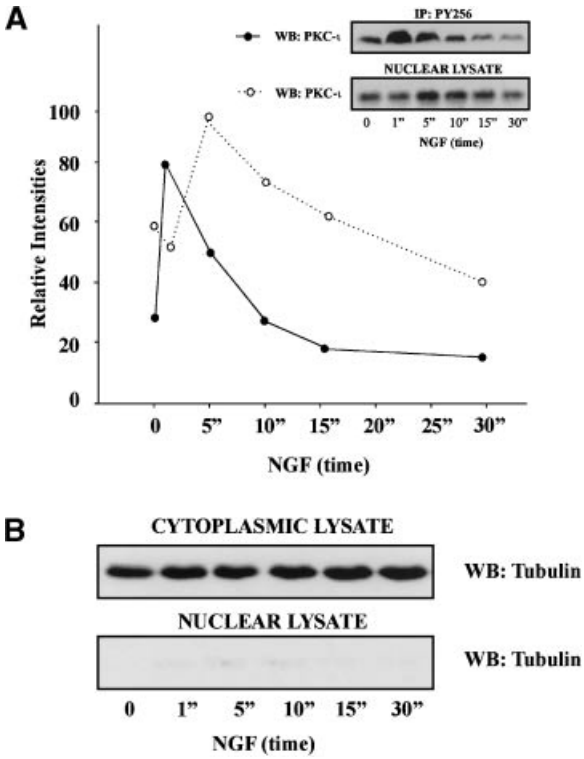

Fig. 3. NGF induces phosphorylation of Tyr256. PC12 cells were stimulated with NGF (50 ng/ml) for indicated times (0–30 min). **A**: The nuclei were isolated, immunoprecipitated with PY256, and Western blots were probed with anti-PKC-ι antibody. Nuclear lysates were loaded at equal concentrations of protein (30 μg) and Western blotted as indicated with PKC-ι. As control both the cytoplasmic and nuclear lysates were blotted as control with anti-tubulin to test for the purity of nuclei. This experiment was performed three times with similar results.

thereby suggesting aPKC may bind to importin-β in the absence of importin-α.

To determine if aPKC employs importin-β to enter the nucleus, GST-importin constructs were used to examine co-association of either importin-α or -β with PKC-ι (Fig. 4A). HEK293 cells were co-transfected with cDNA-HA-PKC-ι in the presence or absence of src. Phosphorylated Tyr256 PKC-ι site-directed mutants [Wooten et al., 2001] were employed to determine if this residue is needed for binding to importin as well. The tyrosine phosphorylation state of PKC-ι was confirmed by anti-HA immunoprecipitation and Western blotting with anti-phosphotyrosine antibody (data not shown). To examine the co-association of PKC-ι with importin, a pull-down assay was employed. Lysates of HEK cells expressing the HA-tagged PKC-ι or mutant Y256F-PKC-ι constructs were added to equivalent amounts of GST-importin α or -β constructs captured on agarose (Fig. 4A). After interaction, the complex was washed extensively in GST-wash buffer, followed by Western blotting employing anti-HA. No interaction between PKC-ι with importin-α was observed (Fig. 4A). Much greater binding with importin-β was observed when PKC-ι was coexpressed with src. However, when the mutated Y256F-PKC-ι construct was included in a GST pull down assay, decreased co-association was observed with importin-β (Fig. 4A). In order to monitor the effects of the Y256F mutation in regulating nuclear import of PKC-ι, PC12 cells were cotransfected with HA-tagged PKC-ι wild type and mutant constructs. Mutation at Y256 abrogated LMB-induced nuclear localization of PKC-ι (Table III). Collectively, these data strongly suggest that src phosphorylated PKC-ι facilitates binding to the nuclear transport protein importin-β regulating entry of PKC-ι into the nucleus.

TABLE III. Localization of Wild-Type and Mutant PKC-ι Protein in Control and NGF Stimulated Cells

	Localization	
Protein	Untreated	+LMB
Wild-type PKC-ι	C	N
Mutant PKC-ι (Y256F)	C	C/NE

PC12 cells were transfected with (1 μg) HA-tagged PKC-ι or HA-tagged mutant PKC-ι Y256F using lipofectamine 2000. Forty eight hours post-transfection, the cells were treated with LMB (4 μM for 1 h), fixed and stained with anti-HA antibodies. C, cytoplasmic; N, nuclear; NE, nuclear envelope.

Importin-β and -α interact through a domain located within the amino terminus of importin α termed the interacting β binding (IBB) domain. This region is essential for dimerization of importin-α with importin-β. Thus, we investigated whether the IBB domain would interfere with the interactions between PKC-ι and importin-β. Competition studies were performed, whereby increasing amounts of His tagged-IBB construct were incubated with GST-importin-β (Fig. 4B). The ability of GST-importin β to bind PKC-ι was then examined by adding lysates from HEK cells expressing pcDNA HA-PKC-ι. The interaction between the proteins was determined by GST-pull down of importin-β and Western blotting for HA-PKC-ι. Increasing concentrations of IBB failed to alter the interaction of importin-β with PKC-ι. Therefore, these data indicate that PKC-ι binds

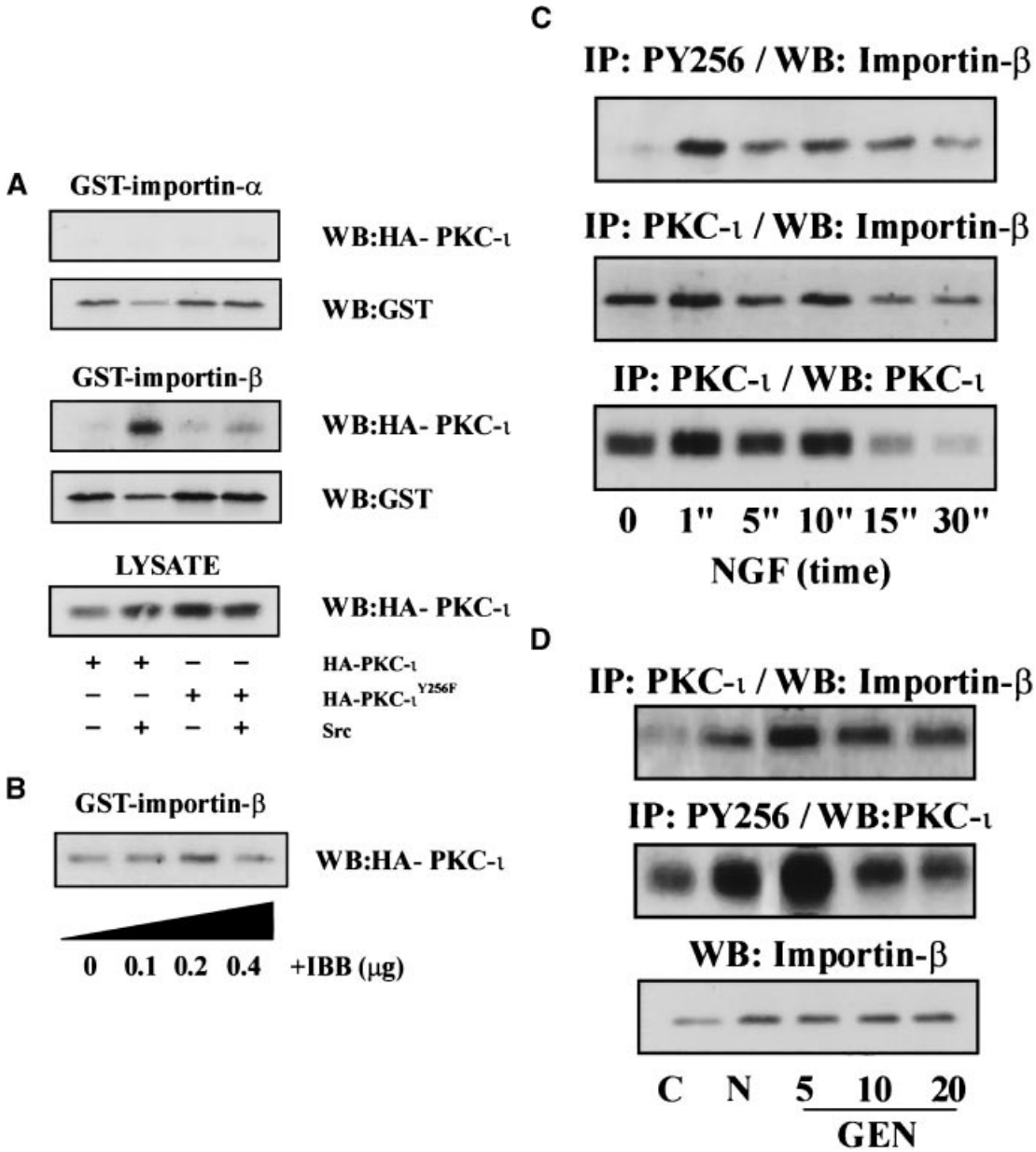

Fig. 4. Phosphorylated Tyr256 PKC-ι is required for binding to importin-β. **A**: HEK293 cells were transfected with pcDNA-HA-PKC-ι or pcDNA-HA-PKC-ι^{Y256F} in the presence or absence of Src as indicated. HEK lysates (250 μg) were included in a pull-down assay with GST-importin-α (10 μg) or GST-importin-α (10 μg). Post interaction the complexes were resolved by SDS–PAGE. The interaction of GST-importin with HA-PKC-ι was determined by anti-HA Western blot analysis. As control, the blots were probed with GST-monoclonal antibody to detect importin-α or β, as indicated. **B**: HIS-IBB peptide does not compete with aPKC-ι binding to importin-β. HEK293 cell lysates (250 μg), prepared from cells transfected with pcDNA-HA-PKC-ι in the presence of Src, were incubated with increasing amounts (0–0.4 μg) of HIS-IBB for 1 h. GST-importin-β (10 μg) was then added to each sample and allowed to rotate for 3 h at 4°C. The GST-importin-β complex was then washed extensively in GST Wash Buffer. The complex was separated by SDS–PAGE followed by Western Blot using anti-HA. **C**: NGF induces co-association of PKC-ι and importin-β in vivo. PC12 cells were treated with NGF for the indicated times (0–30 min). Cell lysates were then immunoprecipitated with either anti-PKC-ι or PY256 antibody followed by Western blot with anti-importin-β or anti-PKC-ι antibody, as indicated. **D**: Inhibition of PKC-tyrosine phosphorylation reduces the co-association of PKC- and importin in vivo. PC12 cells were pre-treated with indicated micro-molar concentrations of genistein: GEN for 1 h followed by 50 ng/ml of NGF for 10 min. The nuclei were isolated by subcellular fractionation followed by immunoprecipitation with anti-PKC-ι or anti-PY256. The samples were then separated by SDS–PAGE and subjected to Western blot using anti-importin-β or PKC-ι, as indicated. As control, the lysates used for immunprecipitation was blotted with importin-β. The blots were scanned and quantitated, genistein was observed to reduce the co-association between PKC-ι and importin-β by 50%.

to importin-β in a non-competitive fashion with importin-α.

NGF treatment of PC12 cells results in nuclear accumulation of aPKCs [Wooten et al., 1997; Zhou et al., 1997; Neri et al., 1999] along with tyrosine phosphorylation [Wooten et al., 2001]. Thus, to determine if NGF-stimulated the interaction of importin-β and PKC-ι in vivo, PC12 cells were stimulated with NGF followed by immunoprecipitation of either PKC-ι or PKC-ι phosphorylated on Y256 and Western blotted for importin-β. Though not dramatic, the difference in the co-association between total PKC-ι and importin-β at 1 min post treatment with NGF compared to control, quantified by scanning densitometry, was about two-fold. However, the difference between PKC-ι binding to importin-β obtained by immunoprecipitation with Ab-PY256 was more than four-fold and paralleled the increase in tyrosine phosphorylated PKC-ι observed in Figure 3. These findings confirm that importin-β has greater affinity for tyrosine phosphorylated PKC-ι than unphosphorylated PKC-ι. Therefore, the interaction of importin-β with PKC-ι in vivo is a NGF-dependent event (Fig. 4C). Altogether, these findings indicate that NGF-induced tyrosine phosphorylation of PKC-ι results in exclusive binding of aPKC's NLS to importin-β, which leads to nuclear import. Since a Y256F mutation in PKC-ι blocks interaction of aPKC with importin-β in vitro, we sought to determine if modulation of the tyrosine phosphorylation state of PKC-ι would effect its interaction with importin-β in vivo. Genistein is a tyrosine kinase inhibitor, that has previously been shown to decrease the tyrosine phosphorylation state of PKC-ι [Wooten et al., 2001]. PC12 cells were pretreated with genistein followed by nuclear isolation (Fig. 4D) and immunoprecipitation with Ab-PY256. Western blotting with importin-β revealed a decreased co-association of PKC-ι with importin-β that paralleled treatment of the cells with the inhibitor. These results further confirm that tyrosine phosphorylation enhances the interaction between aPKC and importin-β.

Model for NGF-Induced Nuclear Translocation of PKC-ι

Based upon these findings, we propose a model for NGF-induced nuclear import of the aPKCs (Fig. 5). Under basal conditions, aPKCs reside in the cytosol in an inactivated form,

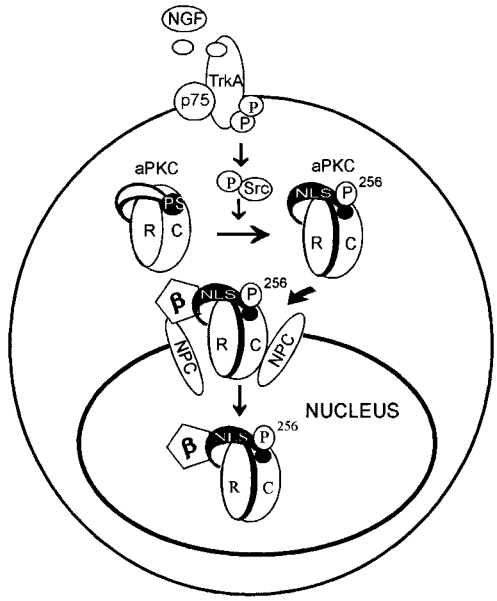

Fig. 5. Model depicting the localization of aPKC in the nucleus. See text for details.

whereby the pseudosubstrate (PS) domain is bound to the substrate binding site. In this conformation, the NLS is buried as a result of the intramolecular interaction between the regulatory and catalytic domain. After NGF stimulation, src phosphorylates aPKC at Tyr256, which induces a conformational change to take place in the PS domain and exposes the NLS motif. Support for the dependence of the PS domain in the functioning of the NLS has been reported [Perander et al., 2001], where interaction between the N-terminal PS sequence and the catalytic domain inhibits nuclear localization. The exposed NLS enables binding of the aPKC exclusively to importin-β, resulting in nuclear import.

DISCUSSION

The study of phosphorylation as a critical mechanism regulating import of proteins into the nucleus has been well-documented [reviewed in Jans et al., 2000]. Various examples exist demonstrating that phosphorylation enhances nuclear import. The interaction of the *Drosophila* transcription factor, Dorsal, with importin is enhanced by phosphorylation [Briggs et al., 1998; Drier et al., 1999]. The adenomatons polyposis coli (APC) protein contains two classical NLSs. Mutation of a

phosphorylation site near the NLS results in an increased import APC into the nucleus [Zhang et al., 2000]. Phosphorylation of residues proximal to the NLS also has been shown to increase nuclear import of the SV40 T-antigen [Hubner et al., 1997]. In the case of cyclin B1, phosphorylation of the cytoplasmic retention signal (CRS) provides the necessary nuclear import signal [Hagting et al., 1999]. Mitogen-activated protein kinase kinase 1(MKK1) is phosphorylated at the lip of the activation loop which regulates entry into the nucleus [Tolwinski et al., 1999].

The importin-α and -β comprise a complex, which facilitate transport across the nuclear pore. The ability of proteins to bind importins have been studied in detail. Interestingly, importin-β binds to arginine rich NLSs [Palmeri and Malim, 1999], whereas, importin-α binds lysine rich NLS-containing proteins. Recently several proteins have been found to bind importin-β directly. The HIV Type 1 Tat and Rev proteins bind to importin-β via a basic arginine-rich NLS [Palmeri and Malim, 1999; Truant and Cullen, 1999]. Likewise, the atypical PKCs contain an arginine-rich bipartite NLS located on the N-terminal side of the zinc finger [Perander et al., 2001], which would enable binding to importin-β. This would explain why aPKC failed to bind importin-α, but selectively binds importin-β. Phosphorylation induces not only a conformational change that opens up the complex, but also enables importin-β to gain high affinity for aPKC, akin to its role in the Smad system [Xiao et al., 2000]. It has been observed that the ability of a protein to bind importin-β is dependent not only on a functional NLS, but also on its appropriate surrounding structure [Xiao et al., 2000]. This is also the case for the binding of aPKC with importin-β.

The kinetics of aPKC-PY256 nuclear import is a rapid event that takes place within 1 min of NGF stimulation. The highest level of nuclear aPKC occurs within 10 min, whereas, NGF induced increases in aPKC activity take place at 12-15 min post NGF stimulation [Wooten et al., 1997; Neri et al., 1999]. In addition, PI3K and PtdIns(3,4,5)P_3 increases in the nucleus occur 10 min post NGF stimulation and activate nuclear aPKC [Neri et al., 1999]. Thus, rapid translocation of aPKC into the nucleus is followed by a subsequent increase in aPKC activity. Therefore, as observed with MKK1 [Zheng et al., 1993], activity of aPKC is not required for nuclear transport. In support of this model, we observed that PKC-ι mutated at Tyr256 is still active [Wooten et al., 2001], yet unable to bind importin-β or enter the nucleus. Altogether, our findings provide a detailed kinetic time frame depicting the entry of aPKC into the nucleus and strengthen the notion that the activity of aPKC is not required for import, but rather occurs as a consequence of localization within the nucleus and the presence of appropriate second messenger.

In addition, the data obtained in this study reveals that dephosphorylation of aPKC may also play a role in regulating export from the nucleus. A Crm1 nuclear export binding motif (NES) located between the zinc finger and the catalytic domain comprising the amino acids 248–255 has been confirmed [Perander et al., 2001]. The identification of both a functional NLS and NES indicates that aPKCs can shuttle between the cytoplasm and the nucleus. The proximity of Tyr256 to the NES suggests phosphorylated Tyr256 might also function to regulate exposure of the NES. Our studies have shown a corresponding increase in Tyr256 with an increase of PKC-ι in the nucleus, whereas, a decrease in Tyr256 occurs concomitant with PKC-ι export from the nucleus, indicating Tyr256 may play a dual role in regulating both import and export. Future studies are needed to further examine the regulation of export via dephosphorylation.

Nucleocytoplasmic shuttling could be hindered by aberrant signaling, which would lead to a decrease in the tyrosine phosphorylation state of aPKCs. Possible mechanisms that result in signaling error due to the altered state of Tyr256 may be lack of phosphorylation of Tyr256, retention of aPKC in the cytoplasm due to an inability to phosphorylate Tyr256, or lack of aPKC export due to inability to dephosphorylate Tyr256. The lack of Tyr256 in the nucleus could impair phosphorylation, and hence the function of aPKC nuclear substrates such as SP1, NF-κB, hnRNPA1, and nucleolin [Zhou et al., 1994; Municio et al., 1995; Pal et al., 1998; Martin et al., 2001], and therefore, have dramatic consequences on cell signaling. Collectively, these studies provide insight into the mechanism, whereby aPKC is imported into the nucleus. This study further underscores the importance of phosphorylation as a mechanism for regulating nuclear transport.

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