Nuclear Import and Export Signals Enable Rapid Nucleocytoplasmic Shuttling of the Atypical Protein Kinase C λ*

Accepted for publication, November 15, 2000, and in revised form, December 12, 2000
Published, JBC Papers in Press, December 13, 2000, DOI 10.1074/jbc.M010356200

Maria Perander†, Geir Bjørkøy, and Terje Johansen§

From the Biochemistry Department, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway

The atypical protein kinase C (PKC) isoenzymes, λ-, and ζPKC, play important roles in cellular signaling pathways regulating proliferation, differentiation, and cell survival. By using green fluorescent protein (GFP) fusion proteins, we found that wild-type λPKC localized predominantly to the cytoplasm, whereas both a kinase-defective mutant and an activation loop mutant accumulated in the nucleus. We have mapped a functional nuclear localization signal (NLS) to the N-terminal part of the zinc finger domain of λPKC. Leptomycin B treatment induced rapid nuclear accumulation of GFP-λPKC as well as endogenous λPKC suggesting the existence of a CRM1-dependent nuclear export signal (NES). Consequently, we identified a functional leucine-rich NES in the linker region between the zinc finger and the catalytic domain of λPKC. The presence of both the NLS and NES enables a continuous shuttling of λPKC between the cytoplasm and nucleus. Our results suggest that the exposure of the NLS in both λ- and ζPKC is regulated by intramolecular interactions between the N-terminal part, including the pseudosubstrate sequence, and the catalytic domain. Thus, either deletion of the N-terminal region, including the pseudosubstrate sequence, or a point mutation in this sequence leads to nuclear accumulation of λPKC. The ability of the two atypical PKC isoforms to enter the nucleus in HeLa cells upon leptomycin B treatment differs substantially. Although λPKC is able to enter the nucleus very rapidly, ζPKC is much less efficiently imported into the nucleus. This difference can be explained by the different relative strengths of the NLS and NES in λPKC compared with ζPKC.

The protein kinase C (PKC) family of lipid-dependent serine/threonine kinases plays pivotal roles in a wide variety of cellular processes (reviewed in Refs. 1–3). Based on sequence homology, domain organization, and biochemical properties, 10 different isoforms are grouped into three classes denoted classical, novel, and atypical PKCs. ζPKC and λ/PKC constitute the atypical PKCs (aPKCs). In contrast to the classical and novel PKCs that contain two repeated diacylglycerol (DAG)-binding zinc finger domains within their regulatory domains, the aPKCs have only a single zinc finger domain that is unable to interact with DAG or phorbol esters (4, 5). Consequently, they do not require DAG for their activation. Atypical PKCs have instead been shown to be regulated in vitro and in vivo by other lipid products such as ceramide (6, 7) and phosphatidylinositol 3,4,5-trisphosphate, a product of phosphatidylinositol 3-kinase (PI 3-kinase) (8–10). Consistently, aPKCs are strongly implicated as downstream effectors of PI 3-kinase (8, 10–14). Recently, evidence has accumulated that imply important roles for aPKCs in processes as diverse as proliferation (15, 16), differentiation (17–19), cell polarity (reviewed in Ref. 20), insulin-mediated up-regulation of general protein synthesis (21), glucose transport (22–24), up-regulation of α2 integrin gene expression (25), and cell survival (26–30).

Interestingly, in addition to cytoplasmic proteins nuclear proteins also have been reported to act as substrates for aPKCs (31–33). The RNA-binding protein nucleolin is phosphorylated by ζPKC in response to nerve growth factor (NGF) treatment of PC12 cells (33). Heterogeneous ribonucleoprotein-A1, another RNA-binding protein involved in splicing and mRNA transport, is also a substrate of ζPKC (31). Both nucleolin and heterogeneous ribonucleoprotein-A1 shuttle between the cytoplasm and the nucleus. The ubiquitously expressed transcription factor Sp1 is able to form a complex with ζPKC. In fact, ζPKC phosphorylates Sp1 within the DNA-binding domain and stimulates Sp1-mediated transactivation of the vascular permeability factor/vascular endothelial growth factor promoter (32). Nuclear localization of both ζ- and aPKC has been demonstrated. NGF stimulation of PC12 cells led to rapid and transient translocation of ζPKC from the cytoplasm to the nucleus (33–35). In resting HepG2 cells ectopically expressed aPKC was found both in the cytoplasm and in the nucleus (38). Upon stimulation with either platelet-derived growth factor or epidermal growth factor, the nuclear pool of aPKC translocated in a wortmannin-sensitive manner to the cytoplasm and to more compact structures within the nucleus.

Specific targeting of signaling protein kinases to subcellular compartments offers an important level of regulation in which the accessibility of their specific activators and substrates can be spatiotemporally limited. The presence of conserved nuclear localization signals (NLSs) allows rapid import to the cell nucleus via the formation of trimeric NLS-importin α-importin β complexes (36–40). During the last few years, short leucine-rich nuclear export signals (NESs) have been identified within a variety of proteins like human immunodeficiency virus-1 Rev (41), PKI (42), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (43), mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-kinase 2) (44), cy-
clin B (45), and phospholipase C-δ1 (46). NES-dependent nuclear export is inhibited by leptomycin B that interferes with the binding of NES to CRM1/exportin 1 (47–51).

Here we have studied the subcellular localization of λ- and γPKC in living cells using green fluorescent protein (GFP) fusion proteins. We find that a kinase-defective mutant of λPKC accumulates in the cell nucleus, whereas the wild-type kinase is mainly cytosolic. Inhibition of CRM1-dependent nuclear export using leptomycin B leads to rapid nuclear accumulation of both GFP-λ and endogenous λPKC. By deletion studies and site-directed mutagenesis, we identified both a functional NLS and an NES in λPKC. These signals endow λPKC with the ability to shuttle continuously between the cytoplasm and the nucleus. Our results are compatible with the notion that the exposure of the NLS in both λPKC and γPKC may be regulated by intramolecular interactions between the N-terminal region and the catalytic domain of the kinases. Also, we find that γPKC is much less efficiently imported into the nucleus than λPKC in HeLa cells upon blockade of nuclear export by leptomycin B treatment. This is most likely due to differences in the relative strengths of the NES and NLS in the two atypical PKCs.

### MATERIALS AND METHODS

**Cell Cultures**—HeLa cells (ATCC CCL2) were grown in Eagle’s minimum essential medium supplemented with 10% fetal calf serum, non-essential amino acids, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Life Technologies, Inc). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, and the antibiotics described above. Subconfluent HeLa and HEK293 cells were transfected using the calcium-phosphate precipitation method. For the nuclear export experiments, leptomycin B, kindly provided by Dr. M. Yoshida, Tokyo, was added to the medium to a final concentration of 2 ng/ml.

**Plasmid Constructions and Site-directed Mutagenesis**—The murine λPKC cDNA was amplified from a mouse brain cDNA library (Marathon Ready, CLONTECH) by PCR using ExTaq polymerase (Takara Biomedicals). The PCR product was made blunt and subcloned into the Smal site of pUC18. Inspection of the cDNA sequences show that both the murine λPKC and the human γPKC cDNAs actually contain an in frame ATG codon nine codons upstream of the proposed start codon (5, 52). The Xenopus λPKC also contains this start codon eight amino acids upstream of the second ATG codon. Since this is the first in frame ATG and the amino acid sequences of this N-terminal extension also are compared between the species, we suggest that the ATG at position 5 is the start codon of λPKC. Consequently, the numbering system used in this paper is based on this. To generate pHA-λ, pUC18-APPKC was digested with EcoRI and XhoI, and the fragment containing the coding region for full-length λPKC (amino acids 1–595) was inserted into the corresponding sites of pcDNA3-HA. pCDNA3-HA was a kind gift from Dr. Jorge Moscat and contains an influenza hemagglutinin (HA) epitope tag inserted into the HindIII-EcoRI sites of pcDNA3 (Invitrogen). pGFP-C1 vector (CLONTECH). pGFP-γ was made from pHA-λ by inserting the EcoRI-XbaI (blunted) fragment encoding pEGFP-C1 vector (CLONTECH). pGFP-γ was made from pHA-λ (kindly provided by Dr. Jorge Moscat) by inserting an EcoRI-XbaI (blunted) fragment encoding the EcoRI-Smal sites of pEGFP-C1 vector. The expressions plasmids for HA-APPKC, GFP-λPKC, and GFP-γ were generated by using the Quick-Change Site-directed Mutagenesis Kit (Stratagene) using the plasmid pAPPKC, pPKC194.5pr and pPKC194.3nt (141–595) was made by PCR using the primers that contained recognition sequences for specific restriction enzymes. The PCR products were purified and digested with restriction enzymes and inserted into the corresponding sites of pEGFP-C1. The constructs were verified by sequencing. All GFP-λ-γ constructs are named according to the included parts of either λPKC or γPKC with the amino acid positions shown in parentheses. To study the localization of the regulatory domain of λPKC, two different constructs were made encoding fusion proteins where λPKC or γPKC was either fused to the N-terminal end or the C-terminal end. pUC18-reg was generated by PCR using pUC18-APPKC as template and the λPKC-5pr and λ256.3nt primers (Table I). The PCR product was made blunt and subcloned into the Smal site of pUC18. pUC18-reg was digested with Nhel (blunted) and EcoRI, and the rega fragment was inserted into the Apal (blunted) and SmaI sites of pEGFP-C1 (CLONTECH). To make pGFP-λ-1–256, pGFP-λ-1–595 was digested with EcoRI and BamHI, and the rega fragment was cloned into the corresponding sites of pEGFP-C1. pGFP-γ-150–595 was made from a PCR product generated by using pUC18-APPKC as template and the primers pAPPKC-5pr and λAPPKC-3nt. The PCR product was cut with XbaI and EcoRI and inserted into the Smal site of pEGFP-C1. pGFP-γ-141–585 was made by PCR using the

| Name | Sequence |
|------|----------|
| AKPCR.5pr | 5'-AGTGGAATTCGCGAGGAGGACGAC-3' |
| AKPCR.3nt | 5'-GAATTCGAGGAGGACGAC-3' |
| AKPKCK285.5pr | 5'-GAGGACACAACACCGGCTTTCGCGGACTCCC-3' |
| AKPKCT411.1E | 5'-GAGGACCAACACCGGCTTTCGCGGACTCCC-3' |
| AKPKCRI50.151E | 5'-GGGCGACAGGGAGGGAGGACGAC-3' |
| AKPKCRI50.151E | 5'-GGGCGACAGGGAGGGAGGACGAC-3' |
| AKPKC253.255A | 5'-TTCGCGAAGGGAGCGAGGGAGGACGAC-3' |
| AKPKCA.129E | 5'-ATGTTGGAATTCGACTTTTCACCGGACGAC-3' |
| AKPKC.5pr | 5'-GTTCGCGAAGGGAGCGAGGGAGGACGAC-3' |
| AKPKCRI50.151E | 5'-GTTCGCGAAGGGAGCGAGGGAGGACGAC-3' |
| AKPKC253.255A | 5'-GTTCGCGAAGGGAGCGAGGGAGGACGAC-3' |
| AKPKCRI50.151E | 5'-GTTCGCGAAGGGAGCGAGGGAGGACGAC-3' |

**TABLE I**

**Sequences of oligonucleotides used as PCR primers for plasmid constructions and site-directed mutagenesis**

---

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
\(\Delta PKC141.5pr\) and the \(\Delta PKC3.3nt\) primers. The product was cut with \(XhoI\) (blunted) and EcoRI and cloned into the EcoRI-SmaI sites of \(pEGFP-C1\). \(pEGFP-C1\) and \((194–256)\) was constructed from a PCR product amplified using the \(\lambda 194.5pr\) and \(\lambda 256.5nt\) primers, digested with \(EcoRI\) and SmaI as described above, and inserted into the EcoRI-SmaI sites of \(pEGFP-C1\). To make \(pEGFP-C1\), the \(\Delta PKC3.3nt\) primers were used, and the PCR product was cut with \(EcoRI\) and \(BamHI\) and inserted into the corresponding sites of \(pEGFP-C1\). To make \(pEGFP-N(37–88)\) and \(pEGFP-(37–155)\), containing one or both of the zinc finger domains from murine \(aPKC\) fused to GFP, the same strategy as for construction of \(pEGFP-(132–182)\) was used except that the \(\Delta PKC37.5pr\) and \(\Delta PKC83.3nt\) primers were used for \(pEGFP-N(37–88)\) and the \(\Delta PKC3.5pr\) and \(\Delta PKC155.3nt\) primers were used for \(pEGFP-N(37–155)\). \(pEGFP-(255–592)\) and \(pEGFP-(310–592)\) were made from PCR products amplified using the \(\Delta PKC\) as template and the \(\Delta PKC3.5pr\) and \(\Delta PKC50.3nt\) primers for \(pEGFP-(252–592)\) and primers \(310.5pr\) and \(310.3nt\) for \(pEGFP-(130–592)\). The PCR products were blunted, cut with \(EcoRI\) and cloned into the EcoRI-SmaI sites of \(pEGFP-C1\). \(pEGFP-(37–182)\) was made from a PCR product generated using the \(\Delta PKC5pr\) and \(\Delta PKC255.3nt\) primers that was cut with \(EcoRI\) and \(BamHI\) and inserted into the corresponding sites within \(pEGFP-C1\). \(pEGFP-(132–182)\) was generated following exactly the same strategy as for \(pEGFP-(251–255)\) using the \(\Delta PKC5.pr\) and \(\Delta PKC182.3nt\) primers for \(pEGFP-(132–182)\) and the primers \(\Delta PKC130.5pr\) and \(\Delta PKC255.3nt\) for \(pEGFP-(130–255)\). Subcellular Localization Analyses and Immunocytochemistry—For the subcellular localization studies of the different GFP fusion proteins, HeLa cells were seeded in 6-well dishes at a density of \(5 \times 10^4\) cells per well 24 h before transfection. The cells were transfected with \(1 \mu g\) of expression vectors. Twenty four h post-transfection the cells were scraped directly in \(100\) of \((2\times\) SDS-polyacrylamide gel electrophoresis load buffer, boiled for \(5\) min, and sonicated briefly. The samples were run on \(10\%\) SDS-polyacrylamide gels and blotted onto Hybond nitrocellulose membranes. The membranes were blocked in \(5\%\) nonfat dry milk in TBST (\(10\) mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for \(1\) h at room temperature and then incubated with the primary antibody diluted in TBST for \(1\) h at room temperature or overnight at \(4^\circ C\). The following primary antibodies were used: anti-GFP, polyclonal (diluted 1:2000, CLONTECH), anti-\(\Delta PKC\) (0.1 \(\mu g/ml\), clone 41, Transduction Laboratories), anti-\(\Delta PKC\) (0.5 \(\mu g/ml\), Upstate Biotechnology, Inc.), and anti-HA (1 \(\mu g/ml\), 12CA5, Roche Molecular Biochemicals). The membranes were washed 6 times in TBST and incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies (0.2 \(\mu g/ml\), Transduction Laboratories) for \(1\) h at room temperature. The washing step described above was repeated, and the membranes were developed using the ECL system following the instructions of the manufacturer (Amersham Pharmacia Biotech). RESULTS Different Subcellular Localization of Wild-type \(\Delta PKC\) and Two Mutants with Single Mutations in the Catalytic Domain—Vectors for expression of murine \(\Delta PKC\) with either an HA epitope tag or enhanced green fluorescent protein (GFP) fused to its N terminus were constructed. The expressed proteins were denoted HA-\(\Delta PKC\) and GFP-\(\Delta PKC\), respectively. A point mutation was introduced into the ATP-binding site to generate a kinase-defective mutant of \(\Delta PKC\), K282W2. To test if the relatively large GFP moiety affected the kinase activity of \(\Delta PKC\), immune complex kinase assays were performed to compare the ability of HA-\(\Delta PKC\) and GFP-\(\Delta PKC\) to phosphorylate histone H1. As shown in Fig. 1, HA-\(\Delta PKC\) and GFP-\(\Delta PKC\) immunoprecipitated from transiently transfected HeLa cells showed little differences in the phosphorylation of histone H1, even being a bit more active than HA-\(\Delta PKC\). The GFP fusion to the CTP-binding site mutant (GFP-K282W) showed no activity as expected. This strongly suggests that the fusion of GFP to the N-terminal of \(\Delta PKC\) has no significant effect on kinase activity. To determine the subcellular localization of \(\Delta PKC\) in living cells, HEK293 and HeLa cells transiently transfected with vectors expressing either GFP-\(\Delta PKC\), GFP-K282W, or GFP alone were analyzed by fluorescence microscopy. GFP-\(\Delta PKC\) was mainly
localized to the cytoplasm in both cell lines although a fraction of the protein was detected in the nucleus (Fig. 2A). Surprisingly, the GFP-λK282W mutant was localized to the cell nucleus in both cell types. The GFP protein alone was distributed diffusely throughout both the cytoplasm and the nucleus. To see if HA-tagged λ or AK282W displayed the same subcellular localization as their GFP counterparts, HEK293 cells were cotransfected with GFP-λ and HA-AK282W or GFP-λK282W and HA-λ. The subcellular localization of HA-λ and HA-λK282W was determined in fixed cells by immunocytochemistry using an anti-HA monoclonal antibody. As shown in Fig. 2B, HA-λ was localized predominantly to the cytoplasm, whereas HA-AK282W accumulated in the nucleus. The nuclear localization of GFP-λK282W was also verified by confocal laser microscopy of transiently transfected NIH 3T3 cells (data not shown). Western blot analyses of whole cell extracts and immunostaining with a monoclonal antibody recognizing specifically the C-terminal catalytic domain of λPKC showed that the nuclear staining was due to full-length protein and not caused by a proteolytic fragment containing GFP and only part of λPKC (data not shown).

Phosphorylation of a conserved threonine residue within the activation loop of all PKCs is crucial for subsequent autophosphorylation and activation of the enzyme (54). Substitution of this threonine in λPKC (Thr-410) with an acidic amino acid created a constitutively activated kinase, whereas replacing it with alanine severely reduced the catalytic activity (13, 55). To test if mutation of the corresponding Thr-411 site in λPKC affected the subcellular localization of the kinase, GFP fusion proteins containing alanine (GFP-λT411A) or glutamate (GFP-λT411E) substitutions at this site were expressed in HeLa cells. The catalytic activity of the T411A mutant was significantly reduced compared with wild-type λPKC and the T411E mutant (Fig. 2C). Interestingly, similar to the kinase-defective ATP-binding site mutant, GFP-λT411A was mainly nuclear in living HeLa cells, whereas GFP-λT411E displayed a predominantly cytosolic localization just as the wild-type λPKC (Fig. 2D). Taken together, these results indicate that the ATP-binding site and the T411A mutations may somehow affect the overall conformation of the protein so that signals governing subcellular localization are exposed differently in these mutants compared with the wild-type enzyme. However, as demonstrated below the observed nuclear accumulation is not correlated to the activity status of the kinase.

The Zinc Finger Domain of λPKC Contains a Nuclear Localization Signal—Proteins larger than about 40–60 kDa cannot enter into the nucleus through the nuclear pore complex by passive diffusion (38, 39). Since both HA-λK282W (67 kDa) and GFP-λK282W (92 kDa) are too large to diffuse into the nucleus, it seemed logical to assume that λPKC could contain a functional nuclear localization signal (NLS) or be transported via interaction with a partner protein containing an NLS. Thus, to map the region(s) of λPKC required for nuclear localization, deletion mutants were made in the context of GFP fusion proteins (Fig. 3A). Plasmids expressing the different deletions were transfected into HeLa cells, and the expression of GFP fusion proteins with correct sizes was verified by Western blot analyses using an anti-GFP antibody (Fig. 3B). Fluorescence microscopy of living cells revealed that GFP-λ(256–595) corresponding to the catalytic domain of λPKC was mainly localized diffusely in the cytoplasm (Fig. 3C). Due to a low level of expression of this construct in HeLa cells, GFP-λ(256–595) was also transiently expressed in HEK293 cells. Compared with the distribution in HeLa cells, GFP-λ(256–595) was even more excluded from the nucleus in HEK293 cells. In contrast, a fusion protein containing the regulatory domain, GFP-λ(1–256) was primarily localized to the cell nucleus. We next analyzed which part of the regulatory domain that was responsible for the observed nuclear accumulation (Fig. 3C). GFP-λ(1–139), containing the first 139 amino acids of λPKC including the pseudosubstrate sequence, was diffusely localized throughout the cell. The molecular mass of this construct is ~42 kDa so the protein will probably enter the nucleus by passive diffusion (38, 39). In contrast, a GFP fusion protein corresponding to the zinc finger domain of λPKC, GFP-λ(141–194), was exclusively localized to the nucleus. This fusion protein further accumulated in structures corresponding to the nucleoli. These observations clearly suggest that the zinc finger domain contains a functional NLS. Surprisingly, GFP-λ(194–256), containing the variable linker region between the zinc finger domain and the ATP-binding site, was excluded from the nucleus. According to the theoretical size of this fusion protein (about 35 kDa) one would expect that it could enter the nucleus by diffusion. Therefore, this fusion protein may be sequestered in the cytoplasm by an anchoring protein, or it may be actively exported from the nucleus.

The sequence identities between the zinc finger domain of λPKC and those of classical and novel PKCs vary from 35 to 48%, whereas there is 74% identity between the zinc finger domains of λPKC and πPKC (Fig. 4A). We therefore asked whether the zinc finger domain of λPKC, like that of πPKC, was able to direct a GFP fusion protein to the nucleus. To this end, HeLa cells were transfected with a construct expressing a GFP fusion protein containing the complete zinc finger domain of murine λPKC, GFP-λ(130–182). Indistinguishable from the results with the corresponding λPKC construct, this fusion protein was exclusively nuclear demonstrating that the zinc finger domain of λPKC also contains an NLS (Fig. 4C). However, GFP fusion proteins containing either the first or both zinc finger domains of the classical isoform αPKC, GFP-α(37–88), and GFP-α(37–155) did not accumulate in the cell nuclei but rather in punctate structures in the cytoplasm (Fig. 4C). Thus, the ability to translocate to the nucleus is not a conserved feature of PKC zinc finger domains.

The zinc finger domain of the αPKCs does not contain classical monopartite or bipartite NLSs. However, λPKC, πPKC, and PKC3 from Caenorhabditis elegans contain a cluster of
Four basic amino acids in the N-terminal part of the zinc finger (KRF/LNRR in Fig. 4A) is not found in the classical and novel PKCs as exemplified by the second zinc finger of δPKC and both zinc fingers of αPKC in Fig. 4A. In a three-dimensional structure model of the zinc finger domain of αPKC, constructed based on the solved structure of the corresponding zinc finger of δPKC, these basic residues are exposed on the surface. Particularly, Arg-150 and Arg-151 are ideally positioned to interact with either lipid cofactors or other proteins (Fig. 4B). To see if introduction of two acidic amino acids in this sequence could interfere with the nuclear localization of kinase-defective αPKC, Arg-150 and Arg-151 were substituted with glutamate residues giving GFP-δPKC containing the complete zinc finger, GFP-(141–162) did not accumulate in the nuclei. Next, we mutated Arg-150 and Arg-151 in the context of the GFP-λ(141–162) protein. GFP-λ(141–162) encoded a GFP fusion protein containing the first 22 amino acids of the zinc finger was made. GFP-λ(141–162) accumulated in the nuclei of transiently transfected HeLa cells (Fig. 4E). In contrast to the GFP fusion protein containing the complete zinc finger, GFP-λ(141–162) did not accumulate in the nuclei as GFP itself (Fig. 4E). Thus, our results strongly suggest that λPKC contains a functional NLS within the first 22 amino acids of the zinc finger domain and that Arg-150 and Arg-151 are critical residues within this NLS.

Leptomycin B Treatment Induces Nuclear Accumulation of λPKC—To determine whether the predominantly cytoplasmic localization of wild-type λPKC is caused by the presence of a leucine-rich NES, GFP-λ-transfected HeLa cells were treated with leptomycin B (LMB). Interestingly, treatment with LMB for 2 h induced nuclear accumulation of the fusion protein (Fig. 5B). To determine whether LMB could induce nuclear accumulation of endogenous λPKC, HeLa cells were either left untreated or treated with LMB for 2 h. The cells were fixed and subcellular localization of endogenous λPKC was determined using immunocytochemistry using a specific anti-λPKC antibody. In untreated cells λPKC was diffusely localized mainly in the cytoplasm with only a fraction of the protein in the nucleus (Fig. 5B, left panel). Importantly, LMB treatment induced redistribution of endogenous λPKC from the cytoplasm to the nucleus (Fig. 5B, right panel). The nuclear accumulation of endogenous λPKC in response to LMB was rapid, being significant after 15 min and almost complete after 30 min (Fig. 5C). These results demonstrate that λPKC is exported from the nucleus by a mechanism either involving a functional cis-acting, LMB-sensitive NES or via an NES-containing interaction partner.

Characterization of an NES within λPKC—Since GFP-λ(194–256), despite of its small size, was completely excluded from the nucleus (see Fig. 3C), we speculated that this part of λPKC, corresponding to the linker region between the zinc finger domain and the ATP-binding site, could be involved in
active export from the nucleus. Interestingly, we identified a region (amino acids 248–255) that displayed significant similarity to previously identified NES sequences (Fig. 6A). To determine whether this region mediates nuclear export of \( \lambda PKC \), we generated a mutant of \( \lambda PKC \) in which two presumably critical hydrophobic amino acids, Phe-253 and Leu-255, are replaced by alanine residues. Similar mutations within NES sequences in other proteins abolish the function of the NES (41–43, 45, 46). In contrast to the wild-type kinase, GFP-\( \lambda F253A/L255A \) accumulated in the nucleus (Fig. 6B). This strongly suggests that cytoplasmic localization of \( \lambda PKC \) is conferred by an NES in the linker region between the regulatory and the catalytic domains.

**Intramolecular Interactions between the N-terminal Pseudosubstrate Sequence and the Catalytic Domain Inhibit Nuclear Localization of \( \lambda PKC \)—**To begin unraveling whether intramolecular interactions between the N-terminal part and the catalytic domain might mask the NLS in \( \lambda PKC \), a GFP-\( \lambda \) fusion protein in which the first 140 amino acids were deleted was transiently expressed in HeLa cells. Contrary to the full-length, wild-type kinase, GFP-\( \lambda (141–595) \) localized exclusively in the cytoplasm (Fig. 7). Since this fusion protein lacks the autoinhibitory pseudosubstrate sequence, the kinase activity of GFP-\( \lambda (141–595) \) was increased compared with the wild-type enzymes (data not shown). To determine whether disruption of the intramolecular interaction between the pseudosubstrate sequence and the catalytic domain led to nuclear localization, a point mutant of GFP-\( \lambda \) in which Ala-129 within the pseudosubstrate sequence was replaced by glutamate, GFP-\( \lambda A129E \), was generated and transiently expressed in HeLa cells. Such a mutant has previously been demonstrated to be constitutively activated presumably due to the lack of interaction of the pseudosubstrate sequence with the substrate interaction site in the catalytic domain (56). Consistent with the notion of a conformational change exposing the NLS, GFP-\( \lambda A129E \) displayed nuclear accumulation (Fig. 7). Taken together, these results and those presented earlier indicate that it is not the activity status of the kinase as such that determines the subcellular localization. Instead, intramolecular interactions between the catalytic domain and the pseudosubstrate sequence inhibit the nuclear localization of \( \lambda PKC \) by inducing a conformation where the NLS is masked.

**Nuclear Import of \( \xi PKC \) Is Much Less Efficient Than That of \( \lambda PKC \) in LMB-treated HeLa Cells—**The \( \alpha PKC \) subtypes, \( \lambda PKC \) and \( \xi PKC \), have the same structural organization and display considerable sequence homology especially within their catalytic domains. To establish if \( \xi PKC \) was similarly distributed within the cell as \( \lambda PKC \), we made a vector expressing GFP fused to full-length \( \xi PKC \), pGFP-\( \xi \). GFP-\( \xi \) was exclusively localized to the cytoplasm in untreated cells. Surprisingly, GFP-\( \xi \) did not accumulate in the nucleus upon LMB treatment for 2 h but was distributed diffusely all over the cell (Fig. 8A). However, after long term treatment with LMB (16 h), GFP-\( \xi \) accumulated in the nuclei of the transfected cells (data not shown). Since we did not have antibodies available that will only recognize \( \xi PKC \) specifically, without detecting \( \lambda PKC \), we were not able to analyze the subcellular distribution of endogenous \( \xi PKC \) by immunocytochemistry. To test whether kinase-defective \( \xi PKC \) localized differently from wild-type \( \xi PKC \), GFP-\( \xi K281W \) mutated in the ATP-binding site was expressed in HeLa cells. Contrary to kinase-defective \( \xi PKC \), GFP-\( \xi K281W \) was predominantly localized in the cytoplasm in a manner similar to wild-type GFP-\( \xi \) (Fig. 8C). Kinase assays performed following immunoprecipitation of GFP-\( \xi \) or GFP-\( \xi K281W \) from extracts of transiently transfected HeLa cells showed that GFP-\( \xi \) was active, whereas GFP-\( \xi K281W \) had no intrinsic kinase activity (Fig. 8C). Similar to GFP-\( \xi \), GFP-\( \xi K281W \) was as active as an HA epitope-tagged \( \xi PKC \) (data not shown). Next, we mutated the putative NES sequence in GFP-\( \xi \) generating GFP-\( \xi P252A/L254A \). Compared with wild-type GFP-\( \xi \), this construct distributed more diffusely all over the cells in transiently transfected HeLa cells but in contrast to GFP-\( \lambda F253A/L255A \), GFP-\( \xi P252A/L254A \) did not accumulate in the nucleus (data not shown). Taken together these results indicate that the nuclear import of \( \xi PKC \) is much less efficient than that of \( \lambda PKC \). To sort out if this is due to intrinsic differences in the relative strength/exposure of the NLS and NES in the two kinases, several GFP-\( \xi \) deletion mutants were made, and their subcellular distribution was compared with the distribution of corresponding GFP-\( \lambda \) mutants. Very interestingly, GFP-\( \xi (130– \)
592) that lacks the first 129 amino acids of γPKC, including the pseudosubstrate sequence, displayed the complete opposite localization compared with the corresponding GFP-γ(141–595) being entirely excluded from the nucleus (Fig. 8D). However, LMB treatment induced rapid nuclear accumulation of this construct. Fig. 9 gives an overview of the subcellular localization of various GFP-γ and γPKC mutants before and after LMB treatment. Our observations indicate that in γPKC the NES is a stronger signal than the NLS, and nuclear localization is only observed when the NES motif is removed or functionally inhibited. In contrast, in γPKC the NES is more potent than the NLS when both signals are exposed, and the nuclear import of γPKC is much more efficient than that of γPKC.

DISCUSSION

Nuclear localization of classical and novel PKCs as well as aPKCs has been observed previously (8, 35, 58–64). However,
to our knowledge, this is the first report where functional NLS and NES sequences are identified within any PKC. We find that λPKC shuttles very rapidly and continuously between the nucleus and the cytoplasm. This rapid nucleocytoplasmic shuttling occurs both in noncycling serum-starved cells and in cycling cells proliferating in serum.

Our results suggest that the core of the NLS of λPKC consists of the hexapeptide KRFNRR located in the N-terminal part of the zinc finger domain (amino acids 146–151). This basic cluster is conserved in aPKCs from different species as well as in C. elegans PKC3 (KRLNRR) but not in classical and novel PKCs (Fig. 4A). An exception is provided by murine ζPKC which contains a Gly residue instead of an Arg (KRFNGR), whereas the rat sequence contains the Arg. GFP fusion proteins containing the zinc finger region of either aPKC or ζPKC (both rat and murine) localize exclusively to the nucleus. In contrast, GFP fusion constructs expressing either one or both zinc fingers of classical aPKC are excluded from the nucleus and rather distributed into punctate structures in the cytoplasm. A recent report (65) demonstrated that GFP fusion constructs that expressed only one or both of the zinc fingers of γPKC localized to the cytoplasm of rat basophilic leukemia cells. Upon treatment with various stimuli including phorbol esters, the zinc finger region of γPKC translocated to the plasma membrane. Thus, although evident for the aPKCs, nuclear localization is not a conserved feature of PKC zinc fingers as such.

Based on sequence analyses it has been suggested that both the classical and the aPKCs may contain a bipartite NLS (35). For λPKC this NLS would encompass two basic amino acids in the pseudosubstrate sequence (Arg-133 and Lys-134) and Arg-150 and Arg-151 in the motif identified by us (KRFNRR) in the zinc finger domain. Two such basic clusters within a bipartite NLS are interdependent on each other to mediate nuclear localization (36). The GFP construct containing the zinc finger region lacks the upstream basic cluster in this suggested bipartite NLS. Since this construct is exclusively localized to the cell nucleus, we do not think that a bipartite NLS is involved in nuclear translocation of λPKC. The KRFNRR motif, although shorter, is most similar to a type of monopartite NLSs enriched in arginine residues identified in the Tat and Rev proteins of human immunodeficiency virus-1 and the Rex protein of human T-cell leukemia virus type 1. These proteins have been demonstrated to be imported into the nucleus by importin β in an importin α-independent manner (66, 67).

We have found that in contrast to wild-type λPKC that mainly localized to the cytoplasm, two different point mutations in the catalytic domain led to nuclear accumulation of full-length λPKC. Nuclear accumulation also occurred with deletion mutants lacking either the catalytic domain or the 140 N-terminal amino acids including the pseudosubstrate sequence. Importantly, the A129E point mutation in the pseudosubstrate sequence, which disrupts the interaction between this autoinhibitory sequence and the substrate interaction site in the catalytic domain, also led to nuclear accumulation of full-length λPKC fused to GFP. The deletion mutant is exclusively nuclear, whereas the point mutant is found also in the cytoplasm. This difference in the extent of relocation relative to the wild-type enzyme is consistent with previous findings showing that regions of the regulatory domain of PKCs outside the pseudosubstrate sequence contribute to autoinhibition (68). We therefore suggest that intramolecular interactions between the catalytic domain and the N-terminal part of the protein regulate the conformation of the protein in such a way that the accessibility of the NLS, the NES, or both signaling sequences is affected. Such a model of regulation has been

![Fig. 7. Intramolecular interactions between the N-terminal pseudosubstrate sequence and the catalytic domain inhibit nuclear localization of λPKC. HEK293 cells transfected with the indicated GFP-λ fusion constructs, and the subcellular localization was determined 24 h following transfection.](http://www.jbc.org/)

![Fig. 8. GFP-ζ does not accumulate in the nucleus following a 2-h treatment with LMB. A, HEK293 cells were seeded in 6-well dishes, and subconfluent cells were transfected with 1 μg of a GFP construct containing wild-type rat ζPKC. Twenty four hours later the subcellular distribution of GFP-ζ was analyzed by fluorescence microscopy in cells which were either left untreated or treated with LMB (2 ng/ml) for 2 h. B, a kinase-defective mutant of ζPKC does not accumulate in the nucleus upon expression in HEK293 cells. GFP-ζ and GFP-ζK281W, which contain an inactivating mutation in the ATP-binding site, were expressed in HEK293 cells. The subcellular localization was determined 24 h post-transfection. C, kinase activity of GFP-ζ and GFP-ζK281W. HEK293 cells were seeded in 100-mm dishes the day before transfection, and subconfluent cultures were transfected with 10 μg of either an expression vector for GFP-ζ or an expression vector for GFP-ζK281W. Cells were harvested 24 h after transfection, and the kinase activities of GFP-ζ and GFP-ζK281W were assayed. Autophosphorylation of immunoprecipitated (IP) GFP-ζ is indicated. A Western blot (WB) of the immunoprecipitated proteins used in the kinase assays is also shown. D, deletion of the N-terminal regulatory domain containing the pseudosubstrate sequence of GFP-ζ does not cause nuclear accumulation in the absence of LMB (2 ng/ml for 2 h).](http://www.jbc.org/)
proteins containing full-length or different parts of both wild-type and mutant will be necessary to determine the three-dimensional structure well documented (69). To understand fully the intramolecular and the substrate interaction site in the catalytic domain is the N-terminal region. An interaction between the pseudosubstrate and the catalytic domain and primarily the exposure of the NLS that is regulated through intramolecular interactions between the catalytic domain and the substrate interaction site in the catalytic domain is well documented (69). To understand fully the intramolecular interactions regulating activity and subcellular localization, it will be necessary to determine the three-dimensional structure of both wild-type and mutant λPKC.

As mentioned in the Introduction, nuclear localization of both λPKC and λPKC has been reported (8, 33, 35). Recently, it was shown that translocation of λPKC to the nucleus following NGF stimulation of PC12 cells probably depends on nuclear PI 3-kinase activity (34). Interestingly, evidence for the existence of nuclear PI 3-kinase activity has been provided (70–72). It has earlier been proposed that conventional PKCs isoforms may continuously shuttle in and out of the nucleus and become “trapped” in the nucleus by an increase in the nuclear level of diacylglycerol (73). In line with this hypothesis, Neri et al. (34) suggest that λPKC is similarly trapped following an increase in nuclear phosphatidylinositol 3,4,5-trisphosphate. Due to their large size a functional NLS is required for nuclear import of aPKCs. We find that the zinc finger domain contains a functional, although atypical, basic NLS. This signal functions independently of a structurally intact zinc finger in the context of a GFP fusion. However, we cannot rule out the possibility that in the context of the full-length protein an intact zinc finger is required for nuclear accumulation. This is particularly the case since the two Arg residues we mutated to Gln resulting in loss of nuclear import may also be involved in the binding of phosphatidylinositol 3,4,5-trisphosphate. Thus, a conformational change may expose the NLS which then enables nuclear import. Subsequently, the protein may become trapped in the nucleus due to binding of nuclear phosphatidylinositol 3,4,5-trisphosphate by the zinc finger.

We find that λPKC is much more inefficiently imported into the nucleus than λPKC upon inhibition of nuclear export. This is in apparent conflict with the work showing rapid nuclear translocation of λPKC following NGF treatment of PC12 cells. However, it is possible that nuclear translocation of λPKC is more tightly regulated than that of λPKC perhaps via post-translational modifications induced by specific stimuli. Another possibility is that both Neri et al. (34) and Wooten et al. (35) are actually looking more at λPKC since the antibodies they used actually recognize both isoforms of aPKCs. We find that PC12 cells express λPKC using a specific monoclonal antibody recognizing only λPKC. However, a similar antibody recognizing only λPKC is not available.

In a recent study Sanchez et al. (57) reported that aPKC colocalized with a putative anchoring protein called p62 into punctate, vesicle-like structures in the cytoplasm corresponding to late endosomes. The concept of p62 serving as an anchoring protein, or perhaps more precisely a scaffolding protein, for aPKCs is very interesting since p62 also seems to be involved in recruiting other proteins into complexes harboring aPKCs (74–76). We have found that, when overexpressed, p62 is able to redistribute kinase-defective λPKC from the nucleus to the cytoplasm and that this ability is dependent on a direct interaction between these two proteins. Thus, it is clear that in addition to regulation of subcellular localization by conformational changes affecting NLS and NES function, the localization of aPKCs is also being regulated by proteins with scaffolding functions such as p62.

Acknowledgments—We are grateful to M. Yoshida for the generous gift of leptomycin B and to J. Moscat for pcDNA3-HA and pHA-κ. We thank T. Lamark for helpful discussions.

REFERENCES

1. Ron, D., and Kazanietz, M. G. (1999) FASEB J. 13, 1658–1676
2. Nishizuka, Y. (1995) FASEB J. 9, 484–496
3. Mellor, H., and Parker, P. J. (1998) Biochem. J. 332, 281–292
4. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3099–3103
5. Akimoto, K., Mizuno, K., Osada, S., Hiraizumi, S., Tanuma, S., Suzuki, K., and Ohno, S. (1994) J. Biol. Chem. 269, 12677–12683
6. Muller, G., Ayahub, M., Storz, P., Rennzecke, J., Fabbro, D., and Pfannesmaier, K. (1995) EMBO J. 14, 1961–1969
7. Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L., and Moscat, J. (1994) J. Biol. Chem. 269, 19200–19202
8. Akimoto, K., Takahashi, R., Moriya, S., Nishioka, N., Takeyanagi, J., Kimura, K., Fukui, Y., Osada, S.-I., Mizuno, K., Hiraizumi, S.-I., Kazlauskas, A., and Ohno, S. (1996) EMBO J. 15, 788–796
9. Nomura, H., Bruzew, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 33–16
10. Standaert, M. L., Galloway, L., Karmam, P., Bandopadhyay, G., Moscat, J., and Farese, R. V. (1997) J. Biol. Chem. 272, 30075–30082
11. Ueno, E., Ueno, J., and Garcia, A. (1997) EMBO J. 16, 5662–5671
12. Herrero-Velit, P., Rutkowski, K. L., and Reiner, N. E. (1997) J. Biol. Chem. 272, 16445–16452
13. Chou, M.-M., Hou, W., Johnson, J., Graham, L.-K., Lee, M.-H., Chen, C.-S., Newton, A.-C., Schaffhausen, B.-S., and Toker, A. (1998) J. Biol. Chem. 273, 1069–1077
14. Takeda, H., Matozaki, T., Takada, T., Noguchi, T., Yamao, T., Tsuda, M., Ochi, F., Fukunaga, K., Inagaki, K., and Kasuga, M. (1999) EMBO J. 18, 386–395
15. Dominguez, I., Diaz-Meco, M. T., Municio, M. M., Berra, E., Garcia de Herreros, A., Coronel, M. E., Sanz, L., and Moscat, J. (1992) Mol. Cell. Biol. 12, 3776–3782
16. Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M., Sanz, L., Lozano, J., Chopkin, R. S., and Moscat, J. (1993) Cell 74, 555–563
17. Coleman, E. S., and Wooten, M. W. (1993) J. Mol. Neurosci. 5, 39–57
18. Wooten, M. W., Zhou, G., Seibenhener, M. L., and Coleman, E. S. (1994) Cell 8, 203–215

* M. Perandér, G. Bjerkøy, T. Lamark, and T. Johansen, manuscript in preparation.
Nuclear Import and Export Signals Enable Rapid Nucleocytoplasmic Shuttling of the Atypical Protein Kinase C λ
Maria Perander, Geir Bjørkøy and Terje Johansen

J. Biol. Chem. 2001, 276:13015-13024.
doi: 10.1074/jbc.M010356200 originally published online December 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M010356200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 77 references, 54 of which can be accessed free at
http://www.jbc.org/content/276/16/13015.full.html#ref-list-1