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# The last five amino acid residues at the C-terminus of PRK1/PKN is essential for full lipid responsiveness☆

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**Abstract**

PRK1/PKN is a member of the protein kinase C (PKC) superfamily of serine/threonine protein kinases. Despite its important role as a RhoA effector, limited information is available regarding how this kinase is regulated. We show here that the last seven amino acid residues at the C-terminus is dispensable for the catalytic activity of PRK1 but is critical for the in vivo stability of this kinase. Surprisingly, the intact hydrophobic motif in PRK1 is dispensable for 3-phosphoinositide-dependent kinase-1 (PDK-1) binding and phosphorylation of the activation loop, as the PRK1-Δ940 mutant lacking the last two residues of the hydrophobic motif and the last 5 residues at the C-terminus interacts with PDK-1 in vivo and has a similar specific activity as the wild-type protein. We also found that the last four amino acid residues at the C-terminus of PRK1 is critical for the full lipid responsiveness as the PRK1-Δ942 deletion mutant is no longer activated by arachidonic acid. Our data suggest that the very C-terminus in PRK1 is critically involved in the control of the catalytic activity and activation by lipids. Since this very C-terminal segment is the least conserved among members of the PKC superfamily, it would be a promising target for isozyme-specific pharmaceutical interventions.


## 1. Introduction

Protein kinase C-related kinase 1 (PRK1, also known as protein kinase N, PKN) is a member of the protein kinase C (PKC) superfamily of serine/threonine protein kinases [1,2]. Like PKC, PRK was originally identified via conventional protein chemistry approaches as a protease-activated serine/threonine protein kinase (PAK) in mammalian tissues [3–5]. Later, molecular cloning of the kinases revealed that this is a group of at least 3 kinases, namely PRK1/PKNα, PRK2/PKNβ and PKNγ [1,2,6]. PRKs are classified as a subgroup of the PKC superfamily as they have a catalytic core highly homologous to that of the PKCs. Numerous studies have implicated PRK1 in cell physiology, such as modulation of cytoskeletal dynamics, cell adhesion, intracellular membranes trafficking and glucose transport as well as its potential involvement in the pathogenesis of cancer and neurodegenerative diseases [2].

The structure of members of the PKC superfamily is highly conserved, with an N-terminal regulator domain linked to a C-terminal catalytic domain via a linker segment (the hinge region). The catalytic core of all PKC superfamily members has 40–50% sequence identity. At the end

*Abbreviations:* aPKC, atypical PKC; cPKC, classic PKC; MD, molecular dynamics; nPKC, novel PKC; PAGE, polyacrylamide gel electrophoresis; PRK1, protein kinase C-related kinase 1; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PDK-1, 3-phosphoinositide-dependent kinase-1; RMSD, root mean square deviation; V3, variable region 3; V5, variable region 5; WT, wild-type.

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of the catalytic domain, there is a C-terminal tail consisting of approximately 70 amino acid residues called the variable region 5 (V5) domain. This V5 domain is present in all PKC/PRKs, ranging from yeast to humans, but with a much lower sequence homology compared with that of the catalytic core [1]. Hence it begs the question of whether this V5 domain of PKC/PRK with seemingly low sequence conservation is essential for the structural integrity and cellular function of the PKC superfamily of kinases.

Despite the overall low homology in the V5 domains among the PKC superfamily members, however, work from a number of laboratories in the last decade has identified two highly conserved motifs in the V5 domains of PKCs: a turn motif (corresponding to $Ser^{920}$ in rat PRK1) and a hydrophobic motif (Phe-Xaa-Xaa-Phe-Ser/Thr(P)-Phe/Tyr, where Xaa is any amino acid). Notably, in isozymes of the atypical PKC and PRK subgroups, the phosphoacceptor Ser/Thr residue is replaced by a negatively charged Asp/Glu residue ($Asp^{940}$ in rat PRK1) [7–9]. Phosphorylation of these two motifs is part of the maturation process that gives rise to functional PKCs, although controversy exists as to the detailed mechanism for the phosphorylation of the hydrophobic motif [10,11]. In addition, phosphorylation of these two motifs contributes to the stability of the PKC isozymes studied and may provide a platform for protein–protein interactions and subcellular targeting of PKCs [7–9]. PKC belongs to the AGC family of serine/threonine protein kinase family [12]. The three-dimensional structure of cAMP-dependent protein kinase A (PKA) is not only the first structure of protein kinase to be solved by X-ray crystallography but also the best characterized structure with information available from both crystal and solution studies. Hence, it serves as the structural prototype for the entire protein kinase family [13,14]. Studies on the conformations of active PKA and protein kinase B (PKB) show that the C-terminal tail (equivalent to the V5 domain in PKCs) of the kinases contributes to substrate recognition and catalysis by helping to secure the kinase core, especially the ATP-binding site, in a functional conformation [15,16]. The hydrophobic motifs of active PKA and PKB are found to localize in a hydrophobic pocket at the back of the catalytic core and make several important contacts with key residues in both N-lobe (small lobe) and the C-lobe (large lobe) of the kinases. However, the sequence of PKA terminates at $Phe^{350}$ (corresponding to $Phe^{939}$ in rat PRK1). Since no crystal structure for the catalytic domain of any of the members of the PKC superfamily is available, several questions remain to be answered: (a) what is the minimal length of the V5 domain that is required for the catalytic competence of PKC/PRKs and for their interaction with upstream kinase 3-phosphoinositide-dependent kinase-1 (PDK-1); and (b) what is the function of the C-terminal extension of PKC/PRK beyond the conserved phenylalanine (corresponding to $Phe^{350}$ in PKA and $Phe^{939}$ in PRK1) in the regulation of this important family of protein kinases.

In this study, we aim to investigate the structure–function relationship of the V5 domain of PRK1. We generated a series of mutants with truncation and/or point mutation in the V5 domain of PRK1 and expressed the mutant PRK1 in mammalian cells. Combined in vitro biochemical analyses and in vivo cell biological assays revealed that the last seven amino acid residues at the C-terminus of PRK1 are dispensable for the catalytic competence of this kinase but are required for the activation of PRK1 by its lipid activator arachidonic acid. We show that, contrary to conventional wisdom, the V5 domain of PRK1 is not required for the solubility in mammalian cells or for the physical interaction with PDK-1.

## 2. Materials and methods

### 2.1. Plasmids and reagents

An N-terminally Myc-tagged rat PRK1 construct was generated by fusing the Myc epitope tag to the second amino acid of rat PRK1 (a generous gift from R.E.H. Wettenhall, University of Melbourne) and subcloning into *Hin*dIII and *Bam*H1 sites of pcDNA3. Monoclonal antibodies to Myc-tag (9E10) were purchased from Roche Diagnostics (Mannheim, Germany). Monoclonal antibodies against PRK1 (Clone 49) and Ha-Ras were obtained from Transduction Laboratories (Lexington, KY). Rabbit and sheep polyclonal antibodies against PDK-1 were from Upstate Biotechnologies. A P500 antibody [17,18] that recognizes the phosphorylated threonine at the activation loop of isozymes of PKC superfamily was a generous gift of A. Newton (University of California, San Diego). Monoclonal antibodies against FLAG-tag (M2), β-actin and all other reagents were from Sigma-Aldrich.

### 2.2. Cell culture and transfections

COS-1 cells were cultured in the high glucose (4500 mg/L) version of Dulbecco's modified Eagle media (Invitrogen) supplemented with 10% fetal calf serum and 2 mM L-alanyl-L-glutamine without antibiotics. Cells were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$ and 95% air. Exponentially growing COS-1 cells (~70% confluence) were transfected in a 35 mm dish using LipofectAMINE (Invitrogen) following procedures recommended by the manufacturer.

### 2.3. Mutagenesis

The truncation and point mutation constructs of PRK1 were generated using a circular plasmid as a template following procedures outlined in Quick-Change® kit (Stratagene). All plasmid DNA were sequenced to verify the presence of desired mutations and that were free of aberrant random mutations. The kinase-dead PRK1 mutant