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Atypical PKC phosphorylates microtubule affinity-regulating kinase 4 in vitro

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Abstract MAP/Microtubule affinity-regulating kinase 4 (MARK4), a Ser/Thr protein kinases, is related to the Par-1 (partitioning-defective) gene products, and is the human ortholog of Par-1. MARK4 shows its role in the cell polarity at the time of embryonic development. It is mostly located at the basal region of cells, providing apico-basal polarity. Here, we made two variants of human Par-1d (MARK4), kinase domain (MARK4-F2), and kinase domain along with 59 N-terminal residues (MARK4-F1) and saw their ATPase hydrolysis in the presence of each other. We observed that the activity of one variant was increased in the presence of other. We also demonstrated that both variants were phosphorylated by atypical PKC and their activities were increased in the presence of increasing concentration of atypical protein kinase c (aPKC). The phosphorylation was observed at the serine and threonine residues of MARK4. The interaction of MARK2 and MARK3 with aPKC and their negative regulation by aPKC is already known. This study confirms a functional link between aPKC and MARK4, two central determinants of cell polarity, and it suggests that aPKC may regulate all four members of Par-1 through phosphorylating them in polarized cells.

Keywords Microtubule affinity-regulating kinase · Cell polarity · Atypical protein kinase C · Par-1 · Microtubule dynamics · ATP

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Introduction

Par-1 (partition-defective), KIN1, and MAP/Microtubule affinity-regulating kinase (MARK) belong to Ser/Thr kinase family and they are protein homolog. They are known to be involved in the cell polarity and also govern cell cycle, cell division, microtubules stability, intracellular signaling, protein stability, etc. [1]. The primary structural organization of these proteins is conserved. All have a catalytic kinase domain, an ATP-binding domain, an ubiquitin-associated domain, and a kinase-associated domain. Among these, the catalytic kinase domain is conserved throughout the evolution [2]. Anteroposterior (A/P) axis formation and germ line determinants polarization are controlled by PAR-1 in nematodes and fruit flies [3], whereas cell wall composition, cell polarization, cell morphology, cell shape, cell growth rate, cell separation, and fission of yeast cells are directly associated by KIN1 [4]. Any defect in the *Caenorhabditis elegans* zygote partitioning is basically due to the mutation in the gene products [5]. MARK4 is a human ortholog of Par-1 [6], which forms a subfamily adenosine monophosphate-activated protein kinases, of the calcium/calmodulin-dependent protein kinase (CAMP) group of kinases [7]. Par3 and Par6 are present only in the apical region of the epithelial cells during embryonic development type cellular events. But MARK4 is mostly found in the basal region of cells, leading apico-basal polarity [8], illustrating its role and significance in the cell polarity.

MARK is known to phosphorylate microtubule-binding domain at the KXGS motif of microtubule-associated proteins (MAPs) [1]. This phosphorylation of MAPs by MARK can disrupt the binding of MAPs to the microtubule and hence change the microtubules dynamics. There are four family members of Par-1/MARK in mammals,

MARK1 (mPar-1c), MARK2 (mPar-1b), MARK3 (mPar-1a), and MARK 4 (mPar-1d) [1, 5, 9–13]. The mammalian kinases LKB1 (Par-4) and MARKK (TAO-1) are the direct upstream activators of MARK in vivo [14–16].

MARK2 is negatively regulated by phosphorylation on threonine residues with atypical protein kinase c (aPKC). This threonine is conserved throughout the evolution in worm, flies, as well as in mammals. MARK3 is also phosphorylated at the equivalent site in vivo and in vitro by aPKC [17]. It is also seen that when MARK2 is phosphorylated on threonine residue in vivo, its plasma membrane localization was negatively regulated [17]. While MARK4 has many interacting partners in cells, it also interacts with aPKC [8]. We made two variants of MARK4, one contains kinase domain along with 59 N-terminal residues known as MARK4-F1, and other has only kinase domain termed as MARK4-F2 for the understanding. Here, we have seen ATPase activity of one variant in the presence of other. In order to know whether MARK4 also gets phosphorylated with aPKC? we tried to phosphorylate both the variants. Our study revealed that MARK4 also gets phosphorylated with aPKC on serine and threonine residues in vitro. This study confirms functional interaction and link between MARK and aPKC, central determinants of cellular polarity. Further, our findings suggest that aPKC phosphorylates MARK on serine and threonine residues and regulates it in polarized cells. We have also observed that the ATPase activity of both the variants get enhanced on phosphorylation with aPKC.

Materials and methods

Luria broth, dialysis tubing, kanamycin, Luria agar, monoclonal anti-His antibody, ampicillin, phosphoserine, and phosphothreonine were purchased from Sigma Chemical Co. (St. Louis, USA). NaCl and EDTA were purchased from Merck (India). Syringe filter of 0.22 μ which is used to filter the protein sample was bought from Millipore Corporation (USA). Ni-HF column was purchased from GE healthcare (GE Healthcare Life Sciences, Uppsala, Sweden). [γ-^{32}P] ATP was purchased from Perkin Elmer (Boston, MA, USA) and aPKC from Promega (Madison, WI, USA). All reagents which were used here are highly pure and are of analytical grade.

Cloning, expression, and purification

We used our well-optimized protocol for cloning, expression, and purification of both the variants of MARK4, MARK4-F1(kinase domain along with 59 N-terminal residues) and MARK4-F2 (kinase domain) and produced both the proteins, as described [18]. Briefly, designed forward and reverse primer was used to amplify both the variants, MARK4-F1 and MARK4-F2. The amplified products were ligated to pQE30 expression vector, and *E. coli* M15 competent cells were used for their transformation. Further, these transformed cells were induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultured overnight at 16 °C. Inclusion bodies (IBs) were processed from this culture and were dissolved in a buffer (CAPS, NaCl, and N lauroylsarcosine). These dissolved IBs buffers were used for proteins purification using Ni–NTA column. Desired MARK4 was eluted with imidazole and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration of the dialyzed and filtered stock solutions was determined experimentally by using molar absorption coefficient of 31775 M^{-1} cm^{-1} for MARK4-F1 and 26150 M^{-1} cm^{-1} for MARK4-F2 at 280 nm [19].

ATPase assays

ATPase assay was done to check the activity of protein in the presence and absence of MARK4-F1 and MARK4-F2. We further checked the ATPase activity after phosphorylation with aPKC. The reaction was incubated for 2 h at 37 °C. Formation of ^{32}P from the hydrolysis of ATP (γ-^{32}P), catalyzed by MARK4-F1 and MARK4-F2 was assayed. Further, this was followed by thin-layer chromatography (TLC), and the quantitation was done by using image-J software as described [20].

Protein phosphorylation

To check whether purified MARK4-F1 and MARK4-F2 are substrate for aPKC, we mixed approximately 200 nM of both the variants of MARK4 in aPKC buffer [10 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM HEPES buffer, 1 mM ATP or 5 μCi [γ-^{32}P] ATP (specific activity 222 TBq/mmol)], and 6 ng aPKC. The reaction was performed at 30 °C for 30 min. Both mixtures were resolved on 12 % SDS-PAGE. These gels were dried and autoradiographed after staining. IMAGE j/geldoc (http://rsbweb.nih.gov/ij/) software was used for their quantification. aPKC was able to phosphorylate both the variants of MARK4 under optimal assay conditions. These phosphorylation reaction mixtures were used for ATPase assays after the incubation was over. For the further experiment, the radioactive-phosphorylated MARK4-F1 and MARK4-F2 bands were excised from these dried gels, dipped in water for hydration, and used for phosphoamino acid analysis. These eluted proteins were further hydrolyzed in HCl (7.5 N) and incubated for 2 h in boiling water bath as described [21]. Finally, centrifugation was done, and supernatants were collected and concentrated. These concentrated supernatants were spotted on

3 mm Whatman chromatography paper with standards phosphoserine and phosphothreonine. In chromatography solution of isopropyl alcohol, NH_4OH (1.0 M) and propionic acid were used in the ratio of 17.5:17.5:45 (v/v). Further, these chromatograms were dried, and stained with ninhydrin solution (0.3 %) before exposed for autoradiography.

Results and discussion

ATPase activity of MARK4 variants is enhanced when present together

The effect of ATPase activity of one variant of MARK4 in the presence of another variant was seen. We observed that when MARK4-F1 and MARk4-F2 are present separately, the percentage hydrolysis is less than when they were together (Fig. 1). It is observed that 200 ng of MARK4-F1 alone could hydrolyze lesser amount of ATP than 100 ng of MARK4-F1 and 100 ng of MARK4-F2 that were taken together (Fig. 1a lane 3 and Fig. 1a lane 5, respectively). In this experiment, BSA (Fig. 1a, lane 1) was used as a negative control. The reason behind this is not very clear, as there is no literature available till now. Some auto-regulation mechanism might be there which increases percentage hydrolysis. Further experiments are needed to know the exact reason.

aPKC phosphorylate MARK4 at serine and threonine residues

In order to know whether aPKC phosphorylation of both the variants of MARK4 was on serine and threonine residues, SDS-PAGE bands of both variants MARK4-F1 and MARK4-F2 after the phosphorylation assay were eluted from the gel. These eluents were hydrolyzed, boiled, centrifuged, concentrated, and followed by the paper chromatography. After staining with ninhydrin solution and autoradiography, the autoradiograms were subjected to phosphoamino acid analysis. Figure 2a, b shows the position of standard phosphoserine and phosphothreonine after staining. After chromatography, these autoradiograms showed the aPKC phosphorylates on serine and threonine residues of MARK4 (Fig. 2). Our observation is supported by the of the report saying that MARK2 and MARK3 got phosphorylated by aPKC on threonine residue [17].

Phosphorylation of MARK4 by protein kinase C

The MARK family has four related proteins, among them MARK2 and MARK3 are phosphorylated by aPKC on threonine residue in vitro [5, 17]. Moreover, MARK4 has many interacting partners in the cells and it also showing interaction with aPKC [8, 22]. Therefore, the phosphorylation of MARK4 protein was checked using aPKC. In order to check the enzymatic activity of both the variants of MARK4 after phosphorylation, the whole reaction mixture of phosphorylation was incubated in the presence of aPKC along with both variants of MARK4 and [γ^{32}P] ATP. Mixtures for both variants resolves on 12 % SDS-PAGE followed by autoradiography. The phosphorylation of proteins was examined by observing an enhanced band in the case of phosphorylation. Bovine serum albumin (BSA) (Fig. 3a, b, lane 1) was used as a negative control in this experiment. Furthermore, it is noteworthy that the MARK4-F1 and MARK4-F2 were phosphorylated with aPKC (Fig. 3a, b). These results clearly indicate that a 35-kDa polypeptide of MARK4-F1 (Fig. 3a, lane 3) and 29-kDa polypeptide of MARK4-F2 (Fig. 3b, lane 3) were phosphorylated by aPKC, considerably. This result shows that like MARK2 and MARK3, MARK4 is also

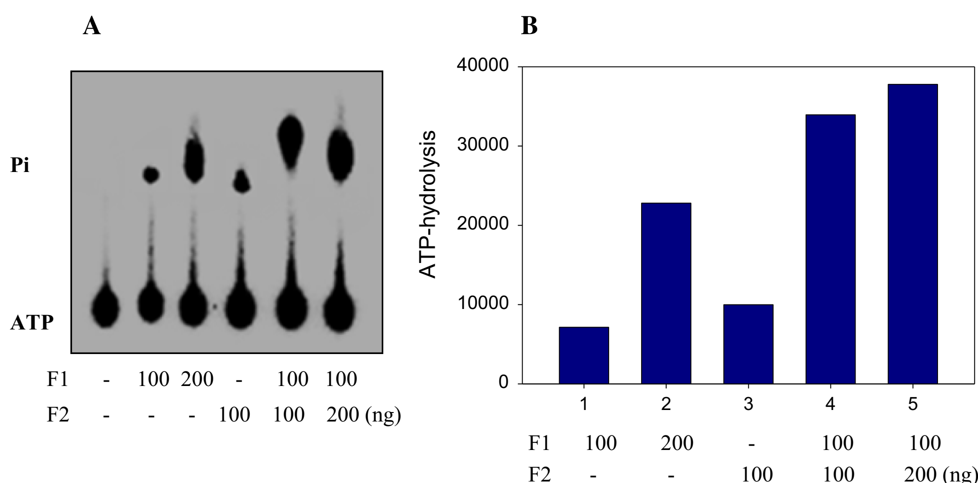

Fig. 1 ATPase activity of MARK4-F1 and MARK4-F2. Positions of Pi and ATP spots are indicated. **a** *Lanes 1*, *2*, *3*, *4*, *5*, and *6* are indicated as control, where there is no protein, 100 ng MARK4-F1, 200 ng MARK4-F1, 100 ng MARK4-F2, 100 ng MARK4-F1 +100 ng MARK4-F2, and 100 ng MARK4-F1 +200 ng MARK4-F2, respectively. **b** ATP-hydrolysis of MARK4 is shown in a *bar* diagram

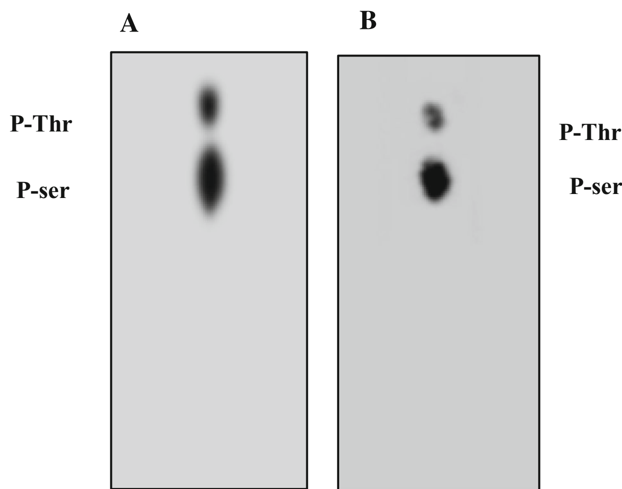

Fig. 2 Phosphoamino acid analysis of **a** MARK4-F1 and **b** MARK4-F2 phosphorylated with aPKC. The positions of standard phosphoamino acids visualized after ninhydrin staining are shown

phosphorylated in vitro by aPKC. Both MARK and aPKC are the key regulator in governing cellular polarity and they show interaction in vivo. There is a functional inter-link between them. We can propose that aPKC may regulate MARKs by phosphorylating it, and hence regulating the cellular polarity and other signaling pathways.

Stimulation of ATPase activities of MARK4 after phosphorylation

ATPase activities of both the variants of MARK4, after phosphorylation by aPKC, were also tested to see the effect of increasing concentration of aPKC on MARK4 phosphorylation. We observed that ATPase (Fig. 4a, b lane 5, 6, 7) activities of both the variants of MARK4 were enhanced after phosphorylation of MARK4-F1 and MARK4-F2 with aPKC as compared to without aPKC phosphorylation (Fig. 4a, b lanes 5, 6, 7 and Fig. 4a, b, lanes 1, 2, 3, respectively). Moreover, The ATPase activities were also increased by increasing the concentration of aPKC. This observation suggests that activity of MARK4 is elevated after phosphorylation by aPKC on serine and threonine residues.

In this study, we identified the phosphorylation of both the variants of MARK4 by aPKC. We demonstrated that the ATPase activities of both variants were increased with increasing concentrations of aPKC. Our finding that MARK4 is phosphorylated by aPKC suggests that MARK4 may be a substrate for aPKC in vivo as interaction between them is shown previously in vivo [8]. We have further shown that aPKC phosphorylates both the variants of MARK4 on the serine and threonine residues

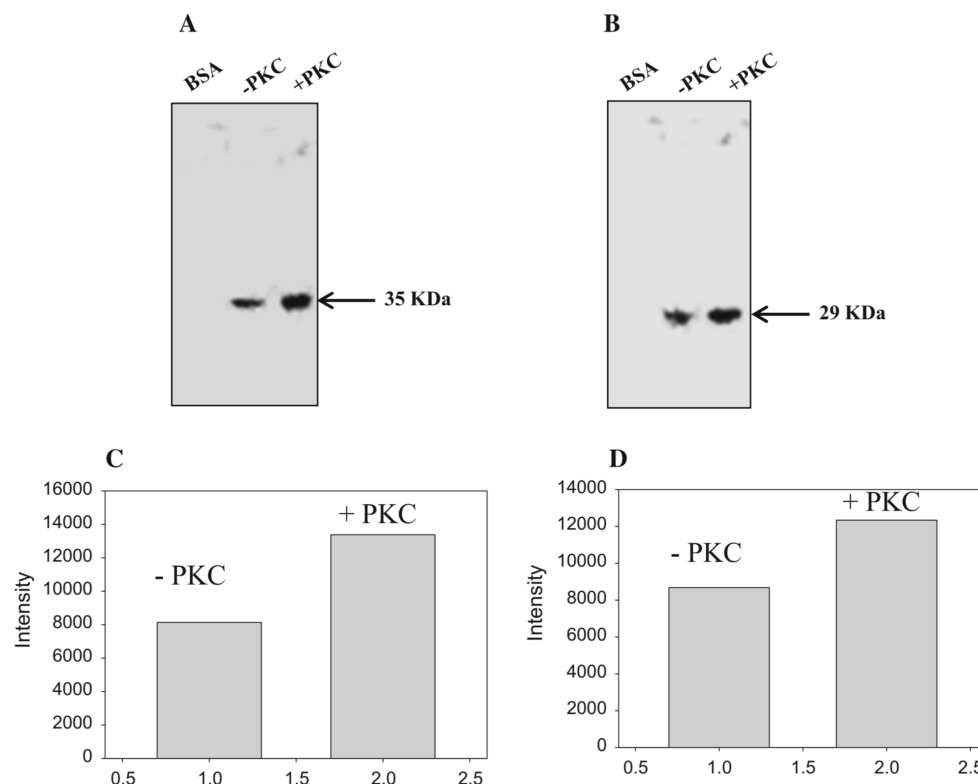

Fig. 3 Phosphorylation of **a** MARK4-F1 and **b** MARK4-F2 with aPKC. *Lane 1* is BSA, *lane 2* is MARK4 without aPKC, and *lane 3* is MARK4 with aPKC. Band intensity of phosphorylated and non-phosphorylated MARK4 is shown in a bar diagram **c** MARK4-F1, **d** MARK4-F2

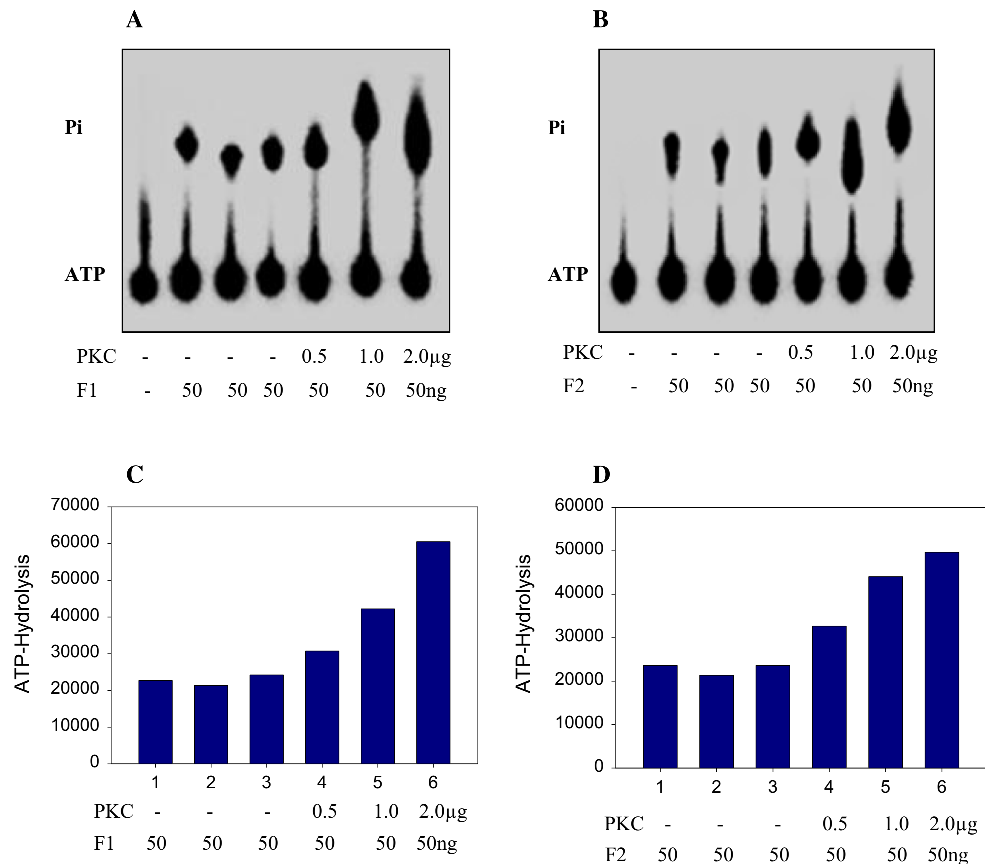

Fig. 4 ATPase activity of **a** MARK4-F1 and **b** MARK4-F2 after phosphorylation with aPKC. *Lane 1*, control without enzyme, *Lane 2* and *Lane 3* are with MARK4, *Lanes 4*, *5,* and *6* are 0.5, 1.0, and 2.0 µg of aPKC. The positions of the inorganic phosphate (Pi) and ATP are marked on the left-hand side of the autoradiograms. ATP-hydrolysis of MARK4 in the absence and presence of aPKC is shown in a *bar* diagram **c** MARK4-F1, **d** MARK4-F2

which are supported by the observation that MARK3 is phosphorylated in vivo on Thr564 and that aPKC phosphorylates MARK2 on Thr564 in vitro [17]. We also demonstrated that the ATPase activity of one variant of MARK4 was increased in the presence of other variant. Although we are not able to describe the reason behind this, we can assume that this may be due to auto-regulation kind of mechanism. Further study is needed to explain the exact mechanism.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest regarding any financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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