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N-terminal PH domain and C-terminal auto-inhibitory region of CKIP-1 coordinate to determine its nucleus-plasma membrane shuttling

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1. Introduction

The pleckstrin homology (PH) domain-containing protein casein kinase 2 interacting protein-1 (CKIP-1) was originally identified as a specific casein kinase 2 (CK2) α subunit-interacting protein [1]. The protein contains a PH domain at the N-terminus, a putative leucine zipper (LZ) motif at the C-terminus, and five proline-rich motifs throughout the protein. However, CKIP-1 was demonstrated to be neither a substrate nor an enzyme regulator of CK2 kinase; instead, CKIP-1 recruits a proportion of nuclear CK2 to the plasma membrane (PM) [2]. Studies based on cultured cells showed that CKIP-1 might participate in regulation of muscle cell differentiation [3], cytoskeleton reorganization [4,5], cell apoptosis [6], and tumor cell growth [7,8]. The physiological role of CKIP-1 in vivo was discovered by the genetic study. We previously showed that

ABSTRACT

The pleckstrin homology (PH) domain-containing protein casein kinase 2 interacting protein-1 (CKIP-1) plays an important role in regulation of bone formation and muscle differentiation. How CKIP-1 localization is determined remains largely unclear. We observed that isolated CKIP-1-PH domain was predominantly localized in the nucleus and the C-terminus of CKIP-1 counteracted its nuclear localization. The net charge of basic residues and a serine-rich motif within the PH domain plays a pivotal role in the localization switch of both full-length CKIP-1 and the isolated PH domain. We propose that the N-terminal PH domain and C-terminal auto-inhibitory region of CKIP-1 coordinate to determine its subcellular localization and the nucleus-plasma membrane shuttling.

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CKIP-1 deficient mice displayed increased osteoblast activity and accelerated bone formation, indicating CKIP-1 physiologically suppressed bone formation [9]. CKIP-1 executes such function through augment of the activity of ubiquitin ligase Smurf1 [9].

PH domains represent the 11th most populous domain family, with \sim 252 examples, in the human proteome [10] and are found in proteins involved in cellular signaling, cytoskeletal organization, and membrane trafficking [11-13]. Although PH domains are previously best known for their ability to bind phosphoinositides with high affinity and specificity, it is now clear that only less than 10% of all PH domains share this property [14,15]. Recent genome-wide analysis of all yeast PH domains and a comprehensive identification of phosphatidylinositol (3,4,5)-triphosphate (PIP3)-regulated PH domains from worm to human both revealed that only a small proportion of the PH domains could either bind to phosphoinositides specifically or be regulated in response to receptor-generated PIP3 production [16,17], and suggested that phosphoinositide binding appears to play an accessory, but not defining, role in targeting these PH domains to membranes. Otherwise, a dual-key strategy has been proposed to argue that the interaction of PH domains with both lipids and proteins on a target membrane are required for efficient membrane targeting [18,19]. Collectively, the functional properties of PH domains are diverse and more complicated than previously thought.

Abbreviations: CKIP-1, casein kinase 2 interacting protein-1; GFP, green fluorescence protein; NLS, nuclear localization signal; RFP, red fluorescence protein; PH, pleckstrin homology; PM, plasma membrane

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Ectopic CKIP-1 localizes predominantly in the PM but also in the nucleus highly dependent of cell types and nutritional status. CKIP-1 is found exclusively in the PM of human breast cancer SK-BR-3 cells [6], but also found in the nucleus of other cell types including osteoblast precursor MC-3T3, myoblast C2C12, and lung carcinoma Glc-82 cells [1,3,20]. Interestingly, CKIP-1 could shuttle between the PM and the nucleus in response to particular stimuli. In C2C12 cells CKIP-1 could translocate from PM to the nucleus under serum-deprivation and return back to the PM after insulin-triggered PI3K activation [3]. CKIP-1 was suggested as a component of PI3K signaling in muscle differentiation [3]. In addition, CKIP-1 could interact with ATM and CK2 kinases, and recruit nuclear ATM and CK2 partially to the PM [2,20]. Nonetheless, the PM localization of CKIP-1 is required for most of its known functions. How the PM localization of CKIP-1 was determined and how the specific shuttling of CKIP-1 between the PM and the nucleus was controlled remains largely unclear.

In this study we surprisingly observed that the isolated PH domain of CKIP-1, in contrast to the full-length CKIP-1, was remarkably nuclear rather than PM-localized independent of cell types. Both the N-terminal PH domain and C-terminal auto-inhibitory region play key roles in control of CKIP-1 PM localization and the nucleus-PM shuttling.

2. Materials and methods

2.1. Plasmid constructs

Plasmids of CKIP-1 and Akt1 including deletion and point mutants were constructed by PCR or recombinant PCR, followed by subcloning into the *Eco*RI/*Kpn*I sites of pEGFP-N1 (Clontech), pDs-Red1-N1 (Clontech) or pFlag-CMV-2 (Sigma) vectors to generate green fluorescence protein (GFP), red fluorescence protein (RFP) or Flag-tagged constructs. Flag-Akt1 was a gift from Drs. Naoya Fujita and Takashi Tsuruo [7] and was used in PCR as the template to generate GFP-Akt1 and GFP-Akt1-PH (1–110). Chimeric fusion constructs CKIP-1-PH-Akt1- Δ PH and Akt1-PH-CKIP-1- Δ PH were generated through interchanging the PH domains. Detailed information of the constructs was available upon request.

2.2. Cell culture, transfections and serum starvation treatment

C2C12, HepG2, NIH3T3, and MCF7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin in a 37 °C incubator with 5% CO₂. The cells were transfected using Lipofectamine 2000 (Invitrogen). For treatment of serum starvation, 24 h after transfection the cells were serum starved by incubation in DMEM alone for 4 h. Stable CKIP-1 knockdown NIH3T3 cells were established as we previously described [6] and cultured in DMEM with G418.

2.3. Immunofluorescence and confocal microscopy

The indicated cells were split onto collagen-coated glass coverslips. After 24 h, the cells expressing Flag or Myc-tagged proteins were washed twice with PBS, and then fixed with 2% paraformaldehyde/PBS for 10 min. After three washes, cells were permeabilized with 3% Triton X-100/PBS for 10 min, incubated with a monoclonal anti-Flag antibody (Sigma, 1:500) or anti-Myc antibody (Clontech, 1:200) for 1 h and then with a corresponding FITC-labeled secondary antibody (1:100) for 1 h. Nuclear staining was performed with DAPI (Sigma). The C2C12, HepG2, NIH3T3 and MCF7 cells expressing GFP or RFP-tagged proteins were visualized with a Zeiss LSM 510 Meta inverted confocal microscope.

2.4. Immunoprecipitation and immunoblotting

GFP or GFP-CKIP-1 plasmids were transfected into the cells as indicated. Forty-eight hours later, cell lysates were prepared in ATM lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% [v/v] Tween-20, 10% glycerol, supplemented with protease inhibitor cocktail [Roche] and phosphatase inhibitors [10 mM NaF and 1 mM Na₃VO₄]) as described [20]. Immunoprecipitations were performed using protein A/G-agarose (Santa Cruz) and anti-CKIP-1 (D-20, Santa Cruz) goat polyclonal antibody to precipitate the full-length CKIP-1 proteins or anti-CKIP-1 (E-18, Santa Cruz) goat polyclonal antibody to precipitate the CKIP-PH truncate at 4 °C. The lysates and immunoprecipitates were detected using the indicated primary antibodies and then the appropriate secondary antibody, followed by detection with SuperSignal chemiluminescence kit (Pierce). GFP antibody was purchased from Cell Signaling Technology, Rabbit polyclonal antibody against phosphoserine (ab9332) was from Abcam. Anti-Myc monoclonal antibody was from MBL.

2.5. Protein-lipid bead binding assay

GST fusion proteins were generated by standard methods. Bead binding assays were performed as described [21]. Briefly, streptavidin–agarose beads bound to biotinylated DiC_6 -PtdIns or –PtdIns(3,4,5)P3 (Echelon) were used to bind and isolate GST-CKIP-PH and GST-CKIP-PH point mutants as indicated. A10 µl slurry of beads was washed once in 500 µl of binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% (v/v) Nonidet P-40) and resuspended in the same buffer, supplemented with 5 µg of GST or GST-CKIP-PH fusion protein, rotating for 2 h at 4 °C. After washing with binding buffer three times, the beads were eluted with Laemmli sample buffer, separated by SDS–PAGE, and immunoblotted with anti-GST (Tiangen) antibody.

3. Results and discussion

3.1. Isolated PH domain of CKIP-1 is localized in the nucleus

To examine the role of the N-terminal PH domain (aa 22–136) in the determination of the CKIP-1 localization, we analyzed the subcellular localization of isolated PH domain. Surprisingly we observed that in contrast to the predominant PM localization of full-length CKIP-1, the single PH domain remarkably distributed in the nucleus and partly in the cytoplasm (Fig. 1A). This phenomenon was also observed in three cell lines derived from different tissues, including C2C12, HepG2 and MCF7 cells (Supplementary Fig. 1). This is unexpected since that the PH domain of CKIP-1 has been demonstrated to bind lipids [2,3,7] and then might recruit the full-length CKIP-1 in the PM. Consistent with previous reports [3,8], RFP-tagged CKIP-1- Δ PH was diffused in the cytoplasm of C2C12 cells (Fig. 1A).

To rule out the possibility of a misleading induced by GFP fusion, a modified RFP-tagged and a short tag Flag-fused PH domain were constructed and examined. Both RFP-CKIP-1-PH and Flag-CKIP-1-PH displayed the same pattern as GFP-CKIP-1-PH did (Fig. 1B). To further verify the nuclear localization of CKIP-1 PH domain, we interchanged the PH domains between CKIP-1 and Akt1 kinase, and generated two fusion proteins. CKIP-PH-Akt1- Δ PH was able to accumulate in the nucleus while Akt1-PH-CKIP- Δ PH appeared in the cytoplasm (Fig. 1C). By comparison, Akt1-WT and Akt1-PH was predominantly PM and secondarily cytoplasm localized (Fig. 1C). Thus the ability of CKIP-1-PH to localize in the nucleus was strong enough to change the distribution of Akt1.

Consistent with a previous study [3], we also observed significant nuclear distribution of CKIP-1 upon serum starvation



Fig. 1. The isolated PH domain of CKIP-1 is localized in the nucleus. (A) Mouse myoblast C2C12 cells were transiently transfected with GFP-tagged CKIP-1, its isolated PH domain (aa 22–136) and RFP-tagged Δ PH (aa 137–409). Twenty-four hours after transfection cells were serum starved in DMEM alone for 4 h. The fluorescence was directly visualized with confocal microscope. Images of representative fields were shown. (B) C2C12 cells were transiently transfected with RFP-tagged or Flag-tagged CKIP-1-PH plasmids. Twenty-four hours later, the localization was determined by confocal microscopy directly or by immunofluorescence using an antibody to Flag. (C) Subcellular localization of the chimeras CKIP-1-PH-Akt1- Δ PH and Akt1-PH-CKIP-1- Δ PH in the presence (left) or absence (right) of serum. Wild-type Akt1 and Akt1-PH domain were also analyzed.

(Fig. 1A, right). Deletion of the PH domain (CKIP-1- Δ PH) (Fig. 1A) or substitution of CKIP-PH with Akt1-PH domain (Akt1-PH-CKIP- Δ PH) (Fig. 1C) retained CKIP-1 in the cytoplasm. By contrast, we did not observe significant nuclear translocation of Akt1 upon serum starvation (Fig. 1C). These data suggested that the PH domain was required for CKIP-1 translocation into the nucleus at least upon serum starvation.

3.2. Identification of a motif within the PH domain critical for its nuclear localization

The classical nuclear localization signal (NLS) is composed of a short stretch of positively charged residues or of a "bipartite" motif, which consists of two basic amino acids, a 10 amino acid spacer and a five amino acid sequence containing at least three basic residues [22,23]. An analysis of the CKIP-1 PH domain revealed a short stretch rich of basic residues, comprising of RKSKSRSKK (aa 82-90) (Supplementary Fig. 2A). The motif is highly conserved across species and composed of six basic residues and three serines which are potentially phosphorylated (Supplementary Fig. 2B). To investigate whether it is involved in determining the nuclear localization of CKIP-1-PH, we constructed a series of point mutants in which the basic residues were mutated to alanines, or the serines were mutated to aspartates, either attenuating or neutralizing the positive charge (Fig. 2A). Compared with the nearly exclusive nuclear localization of wild-type PH domain, the double-point mutants R82A-K83A and K89A-K90A displayed predominantly nuclear distribution but also significant PM localization (Fig. 2A). By contrast, K85A-R87A mutations had no significant alterations compared with the wild-type PH domain, implicating that R82, K83, K89 and K90, but not K85 and R87, play a role in nuclear determination. Consistently, the tetra-point mutant R82A-K83A-K89A-K90A (here named PH-4RKA) was PM-localized without nuclear localization, whereas R82A-K83A-K85A-R87A displayed similar pattern to that of R82A-K83A (Fig. 2A). Mutations of all six basic residues to alanines (PH-6RKA) also relocalized the PH domain to the PM. Intriguingly, substitutions of the three serines to aspartates (PH-S84D-S86D-S88D, named 3SD), mimicking the potential phosphorylation, also displayed the same PM localization to the PH-6RKA and PH-4RKA, strongly indicating that the net positive charges are required for the nuclear localization of CKIP-1 PH domain.

Although PH domains exhibit poor sequence identity, a conserved tryptophan residue was found in the C-terminus of almost all known PH domains and critical for PH domain integrity [14,15]. In CKIP-1 PH domain this residue is W123. W123A mutant was cytoplasm localized and aggregated in speckles (Fig. 2A) [2], which might be caused by inappropriate protein folding. We next tested whether deletion of the RKSKSRSKK motif had any effect on the localization of PH domain. PH- Δ 82-90 was predominantly cytoplasmic similar to PH-W123A (Fig. 2A), implying that the deletion might disrupt the domain integrity.

It has been reported that CKIP-1 PH domain can bind to the phospho-lipids including PtdIns(3,4,5)P3 [7] and W123A mutation resulted in the lipid-binding capability loss [2,7]. To determine whether the nuclear localization of PH domain exhibited any correlation with phospholipid binding, we examined the effects of the PH domain mutations on binding to PtdIns(3,4,5)P3 using

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Fig. 2. Identification of a basic residue and serine-rich motif within the PH domain of CKIP-1 which was critical for its nuclear localization. (A) GFP-tagged CKIP-1-PH (22– 136) and the indicated point mutation and deletion mutant of PH domain were analyzed in C2C12 cells. (B) Protein–lipid bead binding assays. Streptavidin beads coated with either biotinylated PtdIns(3,4,5)P3 or PtdIns were incubated with either GST, GST-PH or GST-PH mutants as indicated for 2 h at 4 °C and then precipitated. Recombinant proteins were eluted, separated by SDS–PAGE, transferred to nitrocellulose filters, and immunoblotted with anti-GST antibody. Input depicts 20% of each recombinant protein that was incubated with the beads. (C) GFP-CKIP-1 mutants 3SD (S84D-S86D-S88D), 4RKA (R82A-K83A-K89A-K90A), W123A, Δ82-90 and 3SA (S84A-S86A-S88A) were transfected into C2C12 cells. Twenty-four hours after transfection, cells were serum starved in DMEM alone for 4 h. The localization of CKIP-1 mutants wisualized with confocal microscopy. (D) Subcellular localization analysis of CKIP-1-PH and CKIP-1-PH mutants in CKIP-1-knockdown NIH3T3 cells. (E) Subcellular localization analysis of CKIP-1 and CKIP-1 mutants in normal cultured and serum-deprived CKIP-1-knockdown NIH3T3 cells. (F) Subcellular localization analysis of CKIP-1-3SA in normal cultured CKIP-1-knockdown NIH3T3 cells. KD represents knockdown.

protein-lipid bead binding assay [21]. Accordingly, GST fusion proteins of wild-type CKIP-1 PH and each of the PH domain mutants (6RKA, 3SD, 3SA and W123A) were expressed in bacteria. Each of these fusion proteins was expressed to comparable degrees. As illustrated in Fig. 2B, wild-type and 6RKA, 3SD, 3SA mutants of CKIP-1 PH domains bound to PtdIns(3,4,5)P3 to similar extents. By contrast, W123A lost this ability as expected and GST alone did not bind to the lipid. The binding to PtdIns(3,4,5)P3 was specific since CKIP-1 PH could not bind to PtdIns (Fig. 2B, right). These results indicate that the effects of RKA, 3SD and 3SA mutations on the localization of CKIP-1 PH domain were not correlated with the ability to interact with phosphoinositide.



Fig. 3. Mapping the auto-inhibitory region (AIR) on CKIP-1. (A) Schematic representation of full-length CKIP-1 and the deletion mutants used to map the AIR. (B–I) Subcellular localization of wild-type (I), the indicated deletion derivatives (B–H) of CKIP-1 expressed as GFP-fusion proteins in C2C12 cells.

3.3. Accumulation of nuclear CKIP-1 after serum starvation is dependent of the ability of the PH domain to be localized in the nucleus

Under culture in normal growth medium, 3SD and 4RKA mutants of full-length CKIP-1 were predominantly PM-localized as well as the wild-type (Fig. 2C). When the cells were serum starved, both CKIP-3SD and -4RKA was still primarily localized in the PM and secondarily in the cytoplasm but not accumulated in the nucleus (Fig. 2C). Since the 3SD and 4RKA mutations abolished the ability of PH domain to be localized in the nucleus, we proposed that CKIP-1 translocation into the nucleus in response to serum starvation might be related to the exhibition of the nuclear localization ability of PH domain. CKIP-1-W123A and CKIP-1- Δ 82-90 were cytoplasmic regardless the status of serum concentration (Fig. 2C), implying that the domain integrity of PH domain might contribute to the nuclear translocation after serum starvation. Furthermore, CKIP-1-S84A-S86A-S88A (3SA) mutant displayed significant nuclear distribution even without serum starvation (Fig. 2C). The results suggested that the charge status of the RKSKSRSKK motif within PH domain was critical in the nuclear translocation of CKIP-1 after serum starvation.

CKIP-1 could form a complex with itself via leucine zipper domain [4,6–8]. To examine whether endogenous CKIP-1 was involved in the regulation of CKIP-1 localization, we detected both the isolated PH domain and the full-length protein upon serum starvation in CKIP-1-knockdown NIH3T3 cells. As shown in Fig. 2D–F, similar results were obtained in these cells compared with those in C2C12 cells, indicating that endogenous CKIP-1 was not involved in the determination of the distribution of CKIP-1 and its mutants. 3.4. The C-terminus of CKIP-1 specifically inhibits the nuclear localization of N-terminal PH domain

The distinct distribution of full-length CKIP-1 and the isolated PH domain clearly indicated that the sequence outside the PH domain must inhibit the nuclear localization of the PH domain and it should specifically exist in CKIP-1 but not Akt1. To attempt to map this auto-inhibitory region (AIR), a series C-terminal truncates were generated (Fig. 3A). CKIP-1 1-337 exhibited significant nuclear and partly extra-nuclear localization. The 1-361 mutant exhibited equal nuclear and extra-nuclear distribution. The 1-382 mutant exhibited obvious PM localization although still retained part nuclear localization. When the mutant was extended to the residue 389 and thereafter, the nuclear localization disappeared and the mutants were localized in the cytoplasm and the PM (Fig. 3C-H). Similar results of these deletion mutants were obtained in two other cell lines HepG2 and MCF7 (Supplementary Fig. 3). These results indicated that the region 382-389 within the C-terminal 72 residues (aa 338-409, we previously called Cterm1 [6]) could function as the proposed AIR (Fig. 3A).

3.5. Overexpression of C-term1 significantly induces nuclear translocation of CKIP-1

The inhibitory effect of CKIP-1 C-terminus on the nuclear localization of PH domain might be direct or indirect. To distinguish these possibilities, an isolated C-term1 was overexpressed together with GFP-CKIP-1. If C-term1/AIR directly inhibits the PH domain, overexpression of C-term1 might have no significant effects or even strengthen the inhibitory effect of the C-terminus



Fig. 4. Ectopic C-term1 induces the nuclear transport of full-length CKIP-1. (A) C2C12 cells were transiently transfected with GFP-CKIP-1 or GFP-Akt1 and Myc-C-term1 plasmids together and visualized 48 h after transfection. Transfections of GFP vector or Myc-vector were used as the controls. (B) The plasmids were cotransfected as in (A). CKIP-1 was directly visualized (green) and C-term1 was visualized through immunostaining with an anti-Myc monoclonal antibody. Cell nuclei were stained with DAPI. (C) C-term1 overexpression induced nuclear transport of GFP-CKIP-1 in a dose-dependent manner. (D and E) Effects of RFP-CKIP-1-ΔPH (D) and RFP-CKIP-1-PH (E) on the localization of full-length GFP-CKIP-1. (F) CKIP-1-ΔPH, CKIP-1-ΔPH, CKIP-1-W123A, CKIP-1-PH, and PH-Δ82-90 proteins were coexpressed with Myc-C-term1 or Myc-vector. Twenty-four hours post-transfection, the GFP fluorescence was visualized. (G) GFP, GFP-CKIP-1, GFP-CKIP-1, GFP-CKIP-1, GFP-CKIP-1, GFP-CKIP-1, GFP-CKIP-1, GFP-CKIP-1, GFP-CKIP-1, GFP-CKIP-1, cell lysates were prepared and immunoprecipitated with anti-CKIP-1 (D-20) antibody. Both the total cell lysates and the immunoprecipitates were subjected by SDS-PAGE and immunoblotted with the indicated antibodies. (H) GFP-CKIP-1 and GFP-CKIP-1 (D-20) and anti-CKIP-1 (E-18) to precipitate full-length and PH domain of CKIP-1, respectively. The immunoprecipitates were analyzed with anti-phospho-serine antibody and the lysates were analyzed with GFP antibody as indicated.

within CKIP-1. If C-term1/AIR indirectly inhibits the PH domain through recruitment of a "second factor", ectopic C-term1 might compete with full-length CKIP-1 to bind to the second factor. Then the inhibitory effect of C-terminus on PH domain should be attenuated and the full-length CKIP-1 should accumulate in the nucleus due to the exposure of PH domain. GFP-CKIP-1

together with Myc-C-term1 or Myc-vector plasmids was transfected into C2C12 cells. As shown, CKIP-1 was mainly localized at the PM when coexpressed with Myc-vector but dramatically redistributed in the nucleus (Fig. 4A) and colocalized with the ectopic C-term1 (Fig. 4B) when coexpressed with Myc-C-term1. These effects could be similarly observed in HepG2 and NIH3T3



cells and CKIP-1-knockdown NIH3T3 cells (Supplementary Fig. 4). By contrast, C-term1 had no significant effect on Akt1 localization (Fig. 4A). Importantly, the ratio of CKIP-1 distribution between the nucleus and the PM was increased companied by the gradual increase amount of the transfected C-term1 plasmid (Fig. 4C), indicating this is dose-dependent. CKIP-1- Δ PH could also recruit CKIP-1 to the nucleus like C-term1 did but the efficiency is less (Fig. 4D). By contrast, CKIP-1-PH had no such effect, although it was in the nucleus (Fig. 4E). Therefore, the C-term1/AIR had a dominant negative effect on the localization of full-length CKIP-1.

To further rule out the possibility that C-term1 recruits CKIP-1 directly from the PM into the nucleus, we examined the effect of C-term1 on other mutants of CKIP-1. C-term1 had no significant effect on the cytoplasmic distribution of CKIP-1- Δ PH, CKIP-1- Δ 82-90, PH- Δ 82-90 and the nuclear distribution of CKIP-1-PH (Fig. 4F). Although W123A could be recruited into the nucleus, the efficiency was lower than that of wild-type CKIP-1 (Fig. 4F). Since Δ PH, W123A and Δ 82-90 contain intact C-terminal part and maintained the ability to interact with C-term1, these results indicated that the direct interaction and recruitment mechanism could be ruled out and suggested the ectopic C-term1 targeted the N-terminal PH domain indirectly rather than interaction with the C-terminus to regulate the nuclear translocation of CKIP-1.

To gain direct insight to see whether CKIP-1 was phosphorylated within the PH domain (especially at S84, S86 and S88) in the presence of serum, GFP-CKIP-1 was immunoprecipitated and detected by immunoblotting with an anti-phosphoserine antibody. As shown in Fig. 4G, phosphorylation of wild-type CKIP-1 but not 3SA (S84A-S86A-S88A) mutant could be detectable in the presence of serum, implicating that these serines could be phosphorylated upon serum stimulation. Importantly, coexpression of Myc-Cterm1 with GFP-CKIP-1 dramatically inhibited the phosphorylation (Fig. 4G). However, the phosphorylation could not be detected neither when the serum was deprived, nor when CKIP-1-PH was expressed even in the presence of serum (Fig. 4H), suggesting that the PH domain alone could not recruit the putative kinase. Instead, the C-terminus was required to recruit the kinase and to regulate the charge status of PH domain.

Taken together, we proposed that the PH domain was required but not sufficient for the localization of CKIP-1 in the PM. Both the PM localization of CKIP-1 under normal culture conditions and the shuttling of CKIP-1 between PM and nucleus require the coordination of N-terminal PH domain and C-terminal autoinhibitory region (AIR). A basic residues and serines-rich motif was critical for the nuclear localization of PH domain and the net charges of the motif can switch PH domain between the PM and the nucleus (Fig. 2). In the presence of the C-terminal AIR, CKIP-1 could be phosphorylated within the PH domain which might inhibit the capability of PH to localize in the nucleus. Upon serum starvation or when the serines were substituted with alanines to prevent the phosphorylation, a proportion of CKIP-1 proteins were relocalized in the nucleus (Figs. 1A and 2C). However, the single PH domain was localized mainly in the nucleus independent of the serum concentration (Fig. 1) and its phosphorylation could not be detectable (Fig. 4H), implying that the C-terminus of CKIP-1 might be critical to recruit the putative kinase. Overexpression of an ectopic AIR/C-term1 might compete with full-length CKIP-1 to bind to the kinase, prevent the PH domain phosphorylation; thus keep the positive charge status within PH domain which triggers CKIP-1 nuclear redistribution (Fig. 4). Although CKIP-1 has been reported to interact with CK2 [1,2], Akt1 [7] and ATM kinases [20], kinase assays showed that CKIP-1 could not be phosphorylated by CK2 [1] and Akt1 [7]. We then tested whether CKIP-1 could be phosphorylated by ATM. CKIP-1 contains four SQ/TQ motifs which are potential phosphorylation sites of ATM (Supplementary Fig. 5A). ATM kinase assays clearly showed that full-length CKIP-1 could be strongly phosphorylated by ionizing radiation-activated ATM kinase in vitro (Supplementary Fig. 5B, left panel, lane 1). As a control, c-Jun could be moderately phosphorylated by ATM (lane 5). By contrast, neither C-term1 (aa 338-409) nor C-term2 (aa 308-409) of CKIP-1 could not be phosphorylated by ATM in vitro (lanes 3 and 4), implying the phosphorylation site might be S277. Nonetheless, we speculated that ATM might be not the candidate kinase responsible for the phosphorylation of CKIP-1 PH domain due to two reasons. One is the PH domain does not possess any SQ/TQ motif and the other is ATM is typically activated by DNA damage and other cellular stresses rather than serum stimulation. The identification of the kinase responsible for PH

Collectively, the coordination of the N-terminus and the C-terminus, but not the PH domain itself, determined the CKIP-1 localization. This dual-key strategy might contribute to further understandings of other PH domain-containing proteins.

domain phosphorylation needs to be performed in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.02.036.

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