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Polo-box motif targets a centrosome regulator, RanGTPase

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Abstract

Mammalian polo-like kinase (Plk) acts at various stages in early and late mitosis. Plk1 localizes in the centrosome, the central spindle, the midbody as well as the kinetochore. The non-catalytic region in the C-terminus of Plk1 has conserved sequence motifs, named polo-boxes. These motifs are important for Plk localization. GFP protein fused with the core sequences of polo-box (50 amino acids) localized Plk to target organelles. We screened for Plk interacting proteins by constructing a tandem repeat of the polo-box motif, and used it as bait in the two-hybrid system with HeLa cell cDNA library. RanGTPase was detected as a positive clone. Through in vitro and in vivo protein binding analysis in synchronized cells by thymidine block and by nocodazole treatment, we confirmed the interaction between endogenous Ran and Plk1. We showed that endogenous Ran and Plk1 proteins were co-localized to centrosomes, which is a major target organelle of endogenous Plk1, in early mitotic cells by immunofluorescence. Finally, we demonstrated that Plk1 phosphorylated RanBPM, a Ran-binding protein in microtubule organizing center, through the interaction with Ran. These data suggested that the core motif of polo-box is sufficient for Plk1-targeting, and that Plk1 may play roles in centrosome through recruitment and/or activation of Ran/RanBPM proteins.

Keywords: Polo-like kinase; Polo-box; Centrosome; RanGTPase; RanBPM

The polo-like kinases (Plks) are conserved subfamily of serine/threonine protein kinases that play numerous roles during M-phase progression [1–3]. Polo kinases are required at several key steps, through initiation of G2/M by phosphorylation of Cdc25C and mitotic cyclins [4–9], centrosome maturation [10,11], and the establishment of a bipolar spindle [8,11,12]. Activation of the anaphase-promoting complex (APC) initiates anaphase and exit from mitosis [13–15]. Microinjection of Plk1 antibodies specific for the C-terminal domain leads to mitotic damage with monopolar spindles, immature centrosomes, and failure of γ-tubulin recruitment to the centrosome [11]. These data suggest a dual role for Plk1 in recruitment and activation of target proteins required for centrosome maturation and bipolar spindle formation. One of the most intriguing features of Plk1 is the dynamic change of its localization and kinase activity during mitosis. Localization of Plk1 to centrosome persists from early mitosis until late anaphase

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[15–20]. Subsequent expression of a recombinant green fluorescent protein (GFP) confirmed the kinetochore/centromere localization of Plk1, suggesting that Plk1 moves to kinetochore and may regulate chromosome and chromatin separation during anaphase [21]. Subsequently Plk1 is found in the central spindle and midbody [15,16,18,19,22].

In addition to the conserved N-terminal catalytic domain, the polo subfamily members are characterized by the presence of conserved motif in the C-terminal non-catalytic domain. Alignment of the C-terminal regions of known several polo-related kinases revealed that there is a motif with significant homology (polo-box) [23]. Mutations in the polo-box of Cdc5, the Plk1 homolog, that do not affect the kinase activity abolish the ability to complement a temperature sensitive mutant of the budding yeast polo-like kinase, Cdc5 [24]. These mutations also abolish the ability for localization to the mitotic apparatus in budding yeast [22,25]. Plo1, the Plk1 homolog in fission yeast, interacts with multiple proteins including cell cycle regulators in Polo-box-dependent manner [26]. In mammalian cells, the C-terminus of Plk1 alone directs to centrosomes and midbody [27,28]. These data suggested that the polo-box is essential motif for biological activity and targeting of Plk in mitosis.

The centrosome is the major microtubule nucleating center and is composed of two barrel-shaped centrioles. Each centriole is formed by nine triplets of microtubules and surrounded by pericentriolar materials [29,30]. Though the centrosome is a small organelle, it directs the nucleation and organization of microtubules, the specification of cell polarity, and the assembly of the bipolar spindle during mitosis [31]. Several protein kinases have been localized in the centrosome and implicated in controlling centrosome function. In addition to Plk1, $p34^{cdc2}$, Aurora-related kinases, and NIMA-related kinase localize in the centrosome either transiently or throughout cell cycle [32]. Cdk2/Cyclin E has been shown to be required for centrosome duplication in cells [33]. Aurora-related kinases are also required for centrosome separation and mitotic spindle assembly [34,35]. One of the important centrosomal components, γ-tubulin, is highly conserved [30,31,36]. Ran is a small GTPase originally identified as a nucleocytoplasmic transporter for large molecules. GTP-bound Ran has been implicated in the control of microtubule assembly [37–40]. A Ran-binding protein, RanBPM, is localized to the centrosome throughout the cell cycle [41], suggesting that Ran may have a role in the centrosome function [42].

Based on the observation that the polo-box is required for localization to centrosome and perhaps other sites, we performed the yeast two-hybrid assay with polo-box motif (50 amino acids) as bait (Fig. 1). In this report, described data suggest that Ran interacts with Plk in the centrosome in mitotic cells.

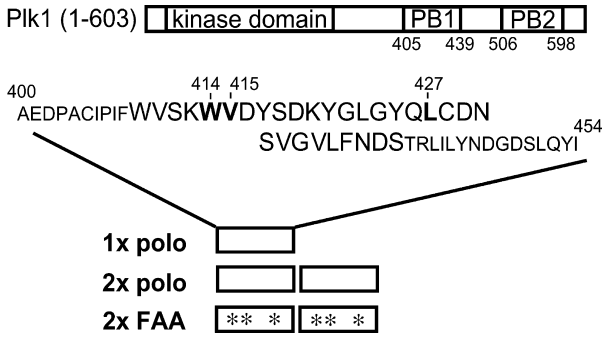

Fig. 1. Schematic representation of polo-box motifs in this study. PB1 and PB2 indicate two predicted polo-boxes in C-terminal region of polo-like kinase1 (residues 405–439 and 506–598) [25]. The 1× indicates the core part of PB1 (residues 400–454). The 2× is a tandem repeated construct of 1 (residues 400–454/400–454). Three point mutations (W414F, V415A, and L427A) were introduced into 2× construct. Bold letters and asterisks indicate the mutated residues.

Materials and methods

Cell culture and mammalian cell transfection. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone) in a humidified incubator at 37 °C. For mitotic arrest, cells were incubated in the presence of 100 ng/ml nocodazole for 16 h. The standard calcium chloride technique of transfection was carried out except for the use of Hepes-buffered saline (HBS) [27,43].

HeLa cells synchronized at the G_1/S boundary were obtained by incubating the cells twice with 2.5 mM thymidine for 16 h with an 8-h release interval. To examine the cell-cycle dependence of Plk1 and Ran1 expression after release from the G_1/S block, cells were washed twice and released into fresh warm DMEM containing 10% calf serum. At the indicated times, cells were collected for Western blot analysis. To determine the cell-cycle stage of the population, flow cytometry was carried out with a FACScan, and data were analyzed by CellFIT-DNA software version 1.2.

Preparation of plasmids. Wild type polo-box motif (residues 400–454) and mutant polo-box were amplified by PCR from pCMV-FLAG-Plk (wild-type) and pCMV-FLAG-Plk (FAA), respectively [27]. The PCR fragments were cloned into pLexA for yeast two-hybrid screening and into pGEX-4T-2 for protein expression in *Escherichia coli*. For subcellular localization, they were cloned into pEGFP-C1 (Clontech) constructs. All constructs were confirmed by DNA sequencing.

In vitro pull-down experiments and immunoprecipitation. GST and MBP proteins were expressed in BL21(DE3) cells. After isopropyl-β-D-thiogalactoside (IPTG) induction for 5 h (0.3 mM final concentration), cells were lysed with PBST buffer (PBS containing 0.5% Triton X-100). MBP fusion proteins were recovered by binding to amylose resins (New England Biolab). Amylose resins were incubated with cell extracts, which contained the GST fusion proteins. After pull down of beads, bound GST proteins were detected by anti-GST antibody. For immunoprecipitation, cells were lysed with 0.5% NP40 buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM Na_3VO_4, 1 mM EDTA, 5 mM EGTA, and protease inhibitor mixture. For the substrates of kinase assay, the immunoprecipitates were washed one more time with kinase buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 2 mM EGTA, and 0.5 mM Na_3VO_4) and applied for kinase assay [44].

Yeast two-hybrid screening. Polo-box motifs were cloned into the pLexA (BD Clontech) in-frame with the LexA DNA-binding domain. The yeast strain, EGY48, carrying reporter gene was cotransformed with the bait plasmid, pLexA-2× (tandem repeat of amino acids

400–405), and a human HeLa cDNA library fused to VP16 activation domain. Transformation was carried out using the lithium acetate method [45]. Leucin-positive colonies were identified by a filter-lifting assay for β-galactosidase activity. Library-derived DNA was prepared from candidate clones and analyzed by DNA sequencing. For triple-hybrid assay, C-terminus of Plk1 was cloned into pGAD424 (Clontech) in-frame with the GAL4-activation domain, and RanBPM and Ran genes were cloned into a pBridge vector (Clontech). RanBPM was cloned in-frame with the GAL4-DNA-binding domain and Ran was located downstream of *Met25* promoter. Transformants were grown in the minimal media with methionine or without methionine.

Immunofluorescence and microscopic experiment. HeLa cells transfected or synchronized were grown on acid-treated coverslips, which were coated with Fibronectin solution (Sigma, F1141), and fixed with 3% paraformaldehyde/methanol [27]. Coverslips were washed three times in PBS, and used for more experiments. For immunofluorescence, goat anti-Ran polyclonal antibody (Santa Cruz Biotech) as primary antibody was used to locate endogenous Ran. A FITC-conjugated mouse anti-goat was used as the secondary antibody. Finally, DNA in cells was detected with propidium iodide (for cells transfected) or DAPI (for immunofluorescence). Following three final washes with PBS signals on coverslips were detected in Zeiss LSM510 confocal microscopy.

Results and discussion

The core polo-box motif of mammalian Plk1 mediates its subcellular localization

The C-terminal region of Plk1 contains highly conserved domains known as polo-boxes: polo-box 1 (PB1) is in amino acids 405–494 and polo-box 2 (PB2) is in amino acids 506–598 [25] (Fig. 1). They contribute for binding to regulate Plk activity as well as for their subcellular localization [13,27,28], and their structure has recently been solved [46,47].

There are several reports on the relationship between polo-boxes and the functions of Plk1. Mutations in PB1 abolished the ability of localization or activation in mammalian or budding yeast [22,25]. Previously, we proposed that C-terminal region of Plk1 is required for auto-inhibition of the catalytic N-terminal domain by intramolecular interaction [27]. This interaction is disrupted by phosphorylation on catalytic domain of Plk1 [44]. Recently, others suggested that a phosphopeptide-binding domain containing two polo-boxes (amino acids 326–603) enables Plk1 to be recruited to substrates, which may be phosphorylated by the priming kinase Cdk1 [48]. In addition, the domain containing PB1 (amino acids 357–603) was required for subcellular localization of Plk1 in U-2OS cells [28]. Because sequences surrounding the polo-box may be important as requirement for an intramolecular folding, we used the core of PB1, which is highly conserved in polo subfamily members, and examined its subcellular localization. In our study, we constructed the core PB1 motif (1× polo, amino acids 400–454) and its tandem repeated construct (2× polo, amino acids 400–454/400–454) (Fig. 1). These constructs were N-terminally linked with EGFP to facilitate subcellular localization in cells and were expressed ectopically in HeLa cells. Asynchronously growing cells were transfected with the DNA constructs of 1× polo, 2× polo, C-terminus of Plk1 (EGFP-C, amino acids 306–603), and 2× polo mutated on the core polo-box (EGFP-2× FAA, amino acids 400–454/400–454 with mutations). Expression of EGFP-1× polo and EGFP-2× polo constructs yielded the distinct fluorescent signals at centrosomes and midbody (data not shown). Recently, Yuan et al. [49] reported that the synthetic core polo-box sequences (amino acids 410–429) linked to an Antennapedia peptide 16-mer for delivery into cells caused mitotic arrest, maligned chromosomes, and multiple centrosome, suggesting that the core motif of PB1 translocated into cancer cell lines induced a similar effect with overexpression of whole C-terminus of Plk1 [27]. Finally, these synthetic polo sequences linked to an Antennapedia peptide inhibited the proliferation of HeLa S3, MCF-7, and Saos-2 cells [49]. These data indicate that the core polo-box motif is sufficient for blocking activity or localization of endogenous Plk1.

The core polo-box motif captures the centrosome regulator, Ran

There have been several reports on the identification of Plk1 interacting proteins by yeast two-hybrid screen

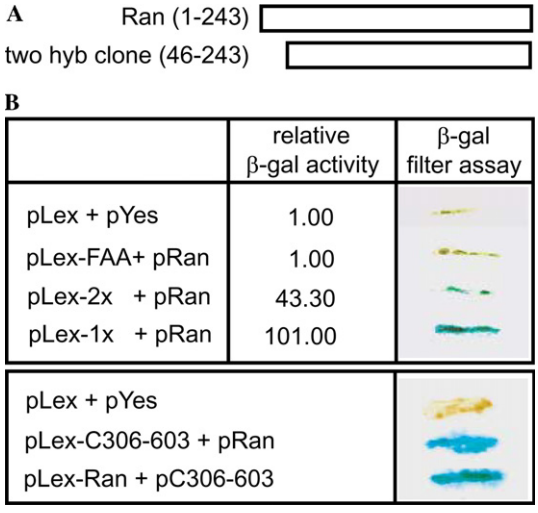

Fig. 2. Ran interacts with the core polo-box motifs by yeast two-hybrid system. (A) After HeLa cDNA library screening, two positive clones coding Ran protein (deleted N-terminal 45 amino acids) were characterized (residues 46–243). (B) The indicated plasmids were transfected into the yeast strain, EGY48. Individual cotransformants were patched onto SD/gal/raf/-His/-Trp/-Leu/-Ura to select for all plasmids expressing interactive hybrid proteins, and β-gal lift assays (see Materials and methods) were performed (β-gal filter assay). Blue color indicates a positive signal. For quantitative analysis of protein interactions, colonies were subjected to liquid β-galactosidase assay as described previously (relative β-gal activity). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

[50–53]. Based on our observations of the specific localizations of the core polo-box sequences, we performed yeast two-hybrid screens of HeLa cell cDNA by using tandem repeated 2× polo motif as bait, which showed stronger localization signal than 1× polo construct (data not shown). In screens for polo-binding proteins, we isolated two clones encoding Ran protein, which regulates mitotic spindle organization and associates with mitotic structures in mammalian cells [38–41,54,55]. The cDNA encoded amino acids 46–243 of Ran (Fig. 2A). In two-hybrid interaction assays for β-galactosidase expression, a full-length construct of Ran and various polo-box motifs, the 1× polo (amino acids 400–454), which are the core sequences of polo-box, showed strong interaction (Fig. 2B, pLex-1× + pRan). The 2× polo, which is tandem repeated construct of core sequences and used for bait for library screen, interacted with full-length of Ran less tightly than 1× polo (Fig. 2B, pLex-2× + pRan). As expected, the 2× polo (FAA) mutated on three conserved amino acids in core polo sequences (W414F/V415A/L427A) does not bind Ran protein (Fig. 2B, pLex-FAA + pRan). As expected, the C-terminal domain containing polo-box motif (amino acids 306–603) also interacted with Ran protein. Then we swapped the vector constructs of bait between C-terminal bait and Ran, and detected their binding (Fig. 2B, pLex-C306-603 + pRan and pLex-Ran + pC306-603).

For in vitro protein binding, the constructs of 1× polo, 2× polo, and C-terminal domain containing intact polo-box were fused to glutathione *S*-transferase (GST). Full-length Ran was fused to maltose-binding protein (MBP). All constructs were expressed in *E. coli* system. Purified MBP-fusion proteins were incubated with the cell extracts that expressed various GST-tagged polo-box constructs (Fig. 3). After incubation, MBP fusion proteins were isolated with amylose beads as previously described, and bound polo-box constructs were detected with anti-GST antibody. Both GST-1× polo and -2× polo interact with MBP-Ran (Fig. 3, upper panel, lanes 1 and 2), whereas GST-2× FAA does not (data not shown). GST-3× polo, which contained a 3-copy tandem repeated polo motif, interacted weakly with MBP-Ran (lane 3). C-terminal domain of 297 amino acids (amino

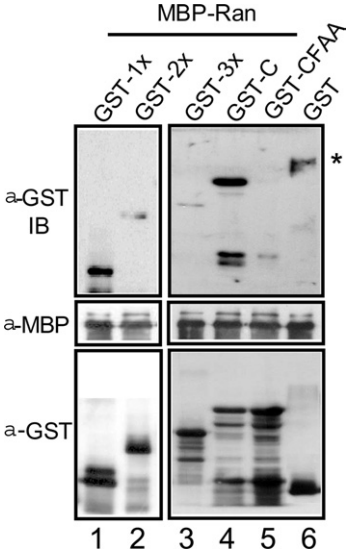

Fig. 3. Polo-box motif interacts with Ran in vitro. GST-fused polo-boxes were expressed in *E. coli*. Cell extracts were incubated with purified MBP-Ran, and pull-downs were probed and detected with anti-GST antibody by Western blotting (upper panel, α-GST IB). The used amounts of MBP-Ran protein purified and GST-polo boxes in cell extracts were estimated by anti-MBP and anti-GST antibodies, respectively (middle and lower panels). Lane 1, incubation of MBP-Ran with cell extract expressed GST-1× polo (single copy of polo motif); lane 2, with cell extract expressed GST-2× polo (two copy tandem repeat); lane 3, with cell extract expressed GST-3× polo (triple copy of polo motif); lane 4, with cell extract expressed C-terminal domain of Plk1 (amino acids 306–603); lane 5, with cell extract expressed C-terminal domain mutated on three amino acids (W414F/V415A/L427A); and lane 6, incubation with GST only. The asterisk indicated non-specific signal. Proteins were subjected onto 15% SDS–PAGE gel.

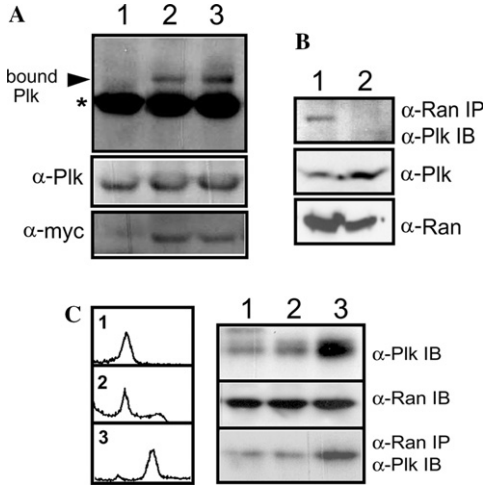

Fig. 4. Endogenous Plk1 interacts with Ran protein in vivo. Interaction between Plk1 and Ran protein showed up in mitotic cells. (A) HeLa cells were transfected with myc-tagged wild-type Ran (lane 2), myc-tagged mutant Ran (L43E) (lane 3), and vector only (lane 1). Nocodazole was treated for mitotic arrest and cells were collected. Myc-tagged Ran proteins were immunoprecipitated by anti-myc antibody. To detect endogenous Plk1 interacting with Ran proteins, immunoprecipitates were probed with anti-Plk1 antibody by Western blotting (upper panel, α-Plk). Amounts of endogenous Plk1 and myc-Ran in mitotic HeLa cell extracts were estimated by anti-Plk1 antibody and anti-myc antibody, respectively (middle and lower panels). (B,C) Interaction between endogenous Plk1 and Ran. (B) Endogenous Ran proteins were immunoprecipitated from unsynchronized cell extract, and Plk1 proteins bound were detected by anti-Plk1 antibody (upper panel). Amounts of both endogenous Plk1 in cell extract and Ran in IP were detected by each antibody (middle and lower panels). Lane 1, immunoprecipitation with anti-Ran antibody; lane 2, cell extract incubated with protein A–agarose without Ran antibody. (C) HeLa cells were arrested at G_1 and at prometaphase by treatment with mimosine and nocodazole, respectively. Cells were harvested and analyzed on cell cycle stages after 16 h (DNA profiles). Upper and middle panels showed the amounts of endogenous Plk1 (α-Plk IB) and Ran1 (α-Ran IB), respectively. Lower panel indicated endogenous Plk1 bound to Ran protein.

acids 306–603) fused to GST interacted with Ran protein fused to MBP, whereas GST-CFAA mutated three amino acids (W414F/V415A/L427A) in polo-box did not (Fig. 3, lanes 4 and 5). There was no specific interaction between GST and MBP-Ran (lane 6). The results of two-hybrid and in vitro binding suggest that the intact core sequences of PB1, which is highly conserved part in subfamily of polo kinase, are important for interaction and localization to cellular targets.

To detect the interaction between Plk1 and Ran in cells, we transfected HeLa cells with myc-tagged wild-type Ran construct and arrested cells in mitotic phase with nocodazole. Cell extracts from mitotic cells were subjected to immunoprecipitation. The proteins were immunoprecipitated with anti-myc-epitope antibody and the presence of associated endogenous Plk1 was determined by Western blot analysis with anti-Plk1 antibody. Specific complex was detected between Plk1 and myc-tagged proteins in mitotic cells (Fig. 4A, upper panel, lane 2). Recently, several groups reported that RanGTP but not RanGDP could induce microtubule aster formation in *Xenopus* CSF arrested egg extracts. One of Ran mutants, RanL43E, maintains Ran in its GTP-bound state and induces aster formation around sperm centrioles. Ran is only present at the centrosome region just after nuclear envelope breakdown in mitosis [38–40]. Thus, RanGTP may interact with endogenous Plk1 after nuclear membrane breakdown. Based on these reports, HeLa cells were transfected with myc-tagged mutant RanL43E, which maintains GTP-bound state. Endogenous Plk1 also interacts with mutant Ran protein (Fig. 4A, upper panel, lane 3).

To investigate the association between endogenous Ran and Plk1, endogenous Ran protein was immunoprecipitated by anti-Ran antibody in unsynchronized HeLa cells, and endogenous Plk1 bound to Ran protein was detected (Fig. 4B, lane 1). Protein A–agarose used in immunoprecipitation did not show any specific binding to endogenous Plk1 (Fig. 4B, lane 2).

To show the difference of protein interaction in cell division stage, HeLa cells were synchronized by treatment with mimosine and nocodazole. Endogenous Plk1 increased in cells arrested in prometaphase (Fig. 4C, panel and lane 3 in α-Plk IB), whereas it decreased in G1 arrested cells (panel and lane 1). Unlike Plk1, the level of endogenous Ran protein was consistent through cell cycle (in middle panel, α-Ran IB). When endogenous Ran was immunoprecipitated by anti-Ran antibody, Plk1 was detected, and its binding was increased as Plk1 level increased (Fig. 4C, lower panel, α-Ran IP/α-Plk IB). These data indicate that endogenous Plk1 interacts with Ran1 in cells.

Because Ran proteins are concentrated in nucleus in interphase (Fig. 5A, middle panels) and is released after break down of nuclear membrane [38–40] (Fig. 5A, lower panels), the interaction between Plk1 and Ran in centromeric region might have occurred in early mitotic phase. To directly examine the locations of Plk1 and Ran, immunofluorescence was performed in HeLa cells. After transfection with RFP-Plk1 construct, cells were fixed and treated with anti-Ran antibody. A FITC-conjugated anti-goat secondary antibody was treated to detect the Ran primary antibody. In interphase, Ran was located in nuclear region, whereas Plk1 was localized at centrosomes (Fig. 5B, upper panels). However, in early mitotic phase, Ran was dispersed into cytoplasm of whole cell, and some were co-localized with Plk1 at centrosomes (Fig. 5B, lower panels). When Plk1 is

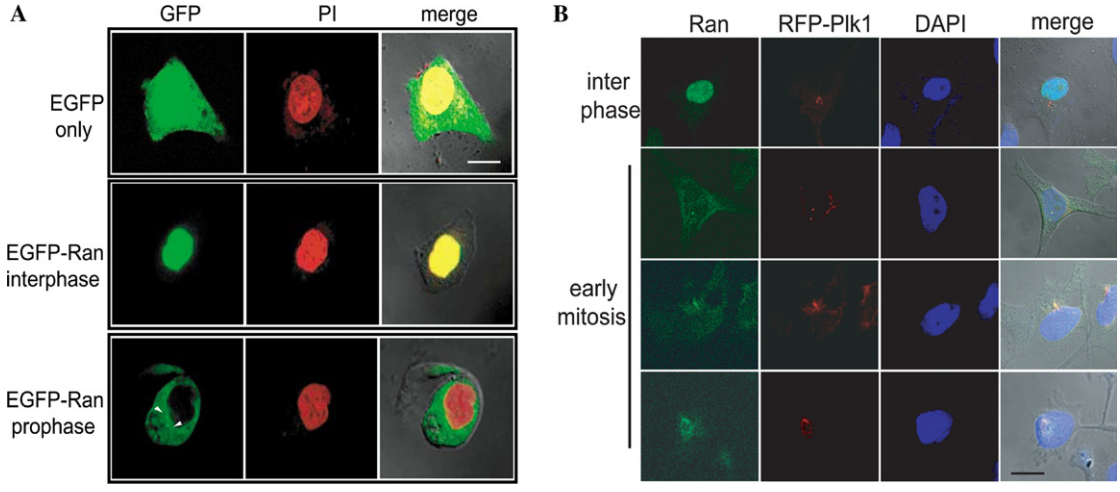

Fig. 5. Subcellular localizations of Plk1 and Ran proteins in HeLa cells. (A) Localization of Ran in interphase and early mitotic phase. HeLa cells were transfected with EGFP vector (upper panels) and EGFP-Ran constructs (middle and lower panels). After treatment of nocodazole, transfectants were fixed and stained with propidium iodide (PI) for DNA. EGFP-Ran was localized in nuclear region in interphasic cells (middle panel, GFP) and released into cytoplasm in early mitotic cells (lower panel, GFP). (B) Endogenous Ran and RFP-Plk1 were co-localized in centrosomes in HeLa transfectants. After transfection with RFP-Plk1 construct, HeLa cells were fixed and stained DNA with DAPI. For detection of endogenous Ran protein, anti-Ran antibody and FITC-labeled secondary antibody were treated as described in Materials and methods. The scale bars represent 10 μm.

overexpressed in HeLa cells, the number of mitotic cells was increased [27]. Indeed, after 30-h of transfection, more than 20% of cells on a coverslip showed that FITC signals were dispersed in whole cell.

Plk1 phosphorylates RanBPM, but not Ran protein

RanBPM, designated as Ran binding protein in microtubule organizing center, has been reported to be as a novel Ran binding protein [41]. RanBPM is localized in the centrosome of either interphase or mitotic cells, and Ran may regulate microtubule aster formation through RanBPM in centrosomal region [54]. Recently, Denti et al. [55] reported that RanBPM is a phosphoprotein and its phosphorylation is regulated by stress stimuli. They implicated that its localization and protein interaction profiles may be regulated by phosphorylation. To investigate whether Plk1 targets RanBPM as well as Ran protein, we performed protein binding and phosphorylation analyses between Plk1 and RanBPM. Although RanBPM does not detectably bind Plk1 (data not shown), Plk1 phosphorylated RanBPM (Fig. 6A, lane 4). Indeed, there was a consensus motif for Plk phosphorylation in the N-terminal region of RanBPM (^{165}DQTVLE) [56]. However, Ran was not phosphorylated by Plk1, even if this protein interacts with Plk1 in vivo and in vitro (Fig. 6A, lane 3). Finally, we showed that the interaction of RanBPM and Plk1 was mediated by expression of Ran protein through yeast triple hybrid assay (Fig. 6B). The cartoon shows the strategy of triple hybrid analysis among Ran, C-terminal domain of Plk1, and RanBPM. The RanBPM fused with GAL4 DNA-binding domain (BD) and Ran protein were expressed in yeast SFY526 strain from

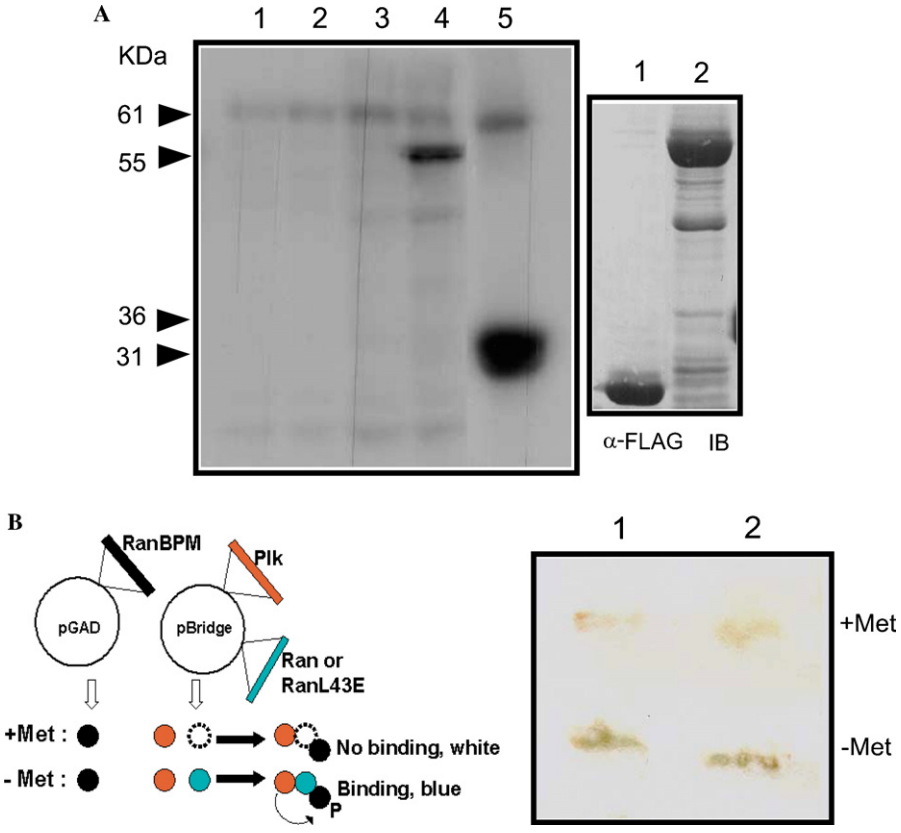

Fig. 6. Plk1 phosphorylates RanBPM protein in vitro and interacts with RanBPM in Ran-dependent manner. (A) HeLa cells were transfected with FLAG-tagged wild-type Ran and RanBPM. Proteins were immunoprecipitated with anti-FLAG antibody from cell extract, and the immunoprecipitates were used as substrates of kinase assay. GST-fused wild-type Plk1 was expressed in Hi5 cells, purified using GST beads, and used as kinase source. Right panel, the amounts of FLAG-tagged proteins in cell extract by Western blot analysis with anti-FLAG antibody (α-FLAG IB). Lanes 1 and 2 indicated FLAG-Ran and FLAG-RanBPM expressed in HeLa cell, respectively. Left panel, phosphorylation signals of immunoprecipitates by Plk1. Lane 1, immunoprecipitates from cells with no DNA; lane 2, immunoprecipitates from cells with pCMV-FLAG vector; lane 3, immunoprecipitates of FLAG-Ran; lane 4, immunoprecipitates of FLAG-RanBPM; and lane 5, 10 μg of dephosphorylated casein as an exogenous substrate. (B) The yeast triple-hybrid analysis among RanBPM, C-terminal domain of Plk1, and Ran (see Cartoon and Result and discussion). Black circle represents RanBPM expressed from pGAD. Red and green circles indicate Plk1 and Ran protein expressed from pBridge vector, respectively. Expression of Ran proteins is regulated by methionine (open circle, no expression in medium containing methionine). For analysis of protein interactions, the plasmids were transfected into the yeast strain, SFY526. Transformants were patched and β-galactosidase lift assays (see Materials and methods) were performed. Lane 1, transformant of pBridge-RanBPM-RanWT and pGAD-PlkC; lane 2, cotransformant of pBridge-RanBPM-RanL43E and pGAD-PlkC. +Met and −Met indicated that yeast cells were grown in the presence of methionine and deficient of methionine, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

the constitutive *ADH1* promoter and conditionally from the *Met25* promoter in response to methionine levels in the medium, respectively. The C-terminus of Plk1 was fused with GAL4-activation domain (AD). When expressions of Ran wild-type or mutant protein (RanL43E) were repressed in the presence of 1 mM methionine, β-galactosidase activity was not detected (Fig. 6B, +Met). Reversely, if Ran proteins are expressed in the absence of methionine, β-galactosidase activity was weakly detected (Fig. 6B, −Met), indicating that C-terminal region of Plk1 might interact with RanBPM in Ran-dependent manner. Because RanBPM fused with GAL4 DNA-binding domain (BD) and C-terminus of Plk1 fused with GAL4-activation domain (AD), which are constitutively expressed in no matter methionine, did not associate with each other (data not shown), β-galactosidase activity was not detected without Ran protein expression (Fig. 6B, +Met in lanes 1 and 2). These data suggested that after nuclear membrane breakdown in early mitosis, Plk1 and Ran protein interact and localize in centrosomal region. In centrosomal region, RanBPM interacts with Ran protein as reported previously [53,54] and is phosphorylated by Plk1, which localized in centrosome. Finally, Plk1 probably target and regulate RanBPM through its Ran-binding for microtubule aster formation in centrosome.

Although centrosome maturation is important for mitotic spindle formation, the underlying mechanisms remain largely unknown. Plk1 has been implicated in the regulation of centrosome maturation [10,11], but the substrate or target of Plk1 is still unknown. Recently, Casenghi et al. [52] reported that Plk1 phosphorylated and regulated centrosome association of nin-ein-like protein (Nlp), which has a role in microtubule organization. Ran protein also regulates microtubule assembly in centrosome region [37–42].

We suggest that the core sequences of PB1 (amino acids 400–454) act as minimal target sequences, and can be used for functional studies of Plk1, and Ran protein, a centrosome regulator, may interact with Plk through this motif in mitotic cells. Moreover, we showed that Plk may interact with Ran protein and this interaction may be important for phosphorylation of RanBPM, which is a key protein of centrosome function in aster formation. More experiments will be performed to understand the significance of the phosphorylation of RanBPM by Plk in centrosomal regulation.

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