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Screening of Domain-specific Target Proteins of Polo-like Kinase 1: Construction and Application of Centrosome/Kinetochore-specific Targeting Peptide

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Mammalian polo-like kinase 1 (Plk1) acts at various stages in early and late mitosis. Plk1 localizes at the centrosome and maintains this position through mitosis. Thereafter Plk1 moves to the kinetochore and midbody region, important sites during chromosome separation and cytokinesis. The catalytic domain of Plk1 is in the N-terminus region, whereas the non-catalytic region in the C-terminus of Plk1 has a conserved motif, named the Polo-box. This motif is critical for Plk localization. EGFP proteins fused with the N-terminus and C-terminus of Plk1 localize in the nucleus and centrosomes, respectively. The core sequences of the polo-box (50 amino acids) also localize in Plk1 target organelles. To screen for domain-specific target proteins of Plk1, we constructed an N-terminal domain and a tandem repeat polo-box motif, and used them as templates in a yeast two-hybrid screen. The HeLa cell cDNA library indicated several proteins including the centrosome/kinetochore components or regulators, to be characterized as positive clones. Through *in vitro* protein binding analyses, we confirmed an interaction between these proteins and Plk1. The data reported from this study indicate that the N- and C-termini of Plk1 may function through recruitment and/or activation of domain-specific target proteins in dividing cells. Additionally, tandem repeats of the conserved core motif of the polo-box are sufficient for targeting and may be useful as a centrosome/kinetochore-specific targeting peptide.

Keywords: Centrosome, Kinetochore, Polo-box motif, Polo-like kinase 1, Yeast two-hybrid screening

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Introduction

The polo-like kinases (Plks) are a conserved subfamily of serine/threonine protein kinases that play numerous roles during M-phase progression (Glover *et al*., 1996; Donaldson *et al*., 2001). Polo kinases are required at several key steps in the process, through initiation of G2/M by phosphorylation of Cdc25C and mitotic cyclins (Abrieu *et al*., 1998; Qian *et al*., 1998; Karaiskou *et al*., 1999; Toyoshima-Morimoto *et al*., 2001), centrosome maturation (Sunkel and Glover, 1988; Lane and Nigg, 1996), and the establishment of a bipolar spindle (Ohkura *et al*., 1995; Lane and Nigg, 1996; Qian *et al*., 1998). Activation of the anaphase-promoting complex (APC) initiates anaphase and an exit from mitosis (Descombes and Nigg, 1998; May *et al*., 2002). Microinjection of Plk1 antibodies specific for the C-terminal domain leads to mitotic damage with monopolar spindles, immature centrosomes, and failure of g-tubulin recruitment to the centrosome (Lane and Nigg, 1996). The C-terminal domain of Plk1 inhibits Plk1 kinase activity through intramolecular interaction, and the overexpression of this domain causes centrosome abnormalities (Jang *et al*., 2002a). Recently, phenotype analysis based on an RNA interference approach revealed the essential function of Plk1 for cell proliferation and viability (Spankuch-Schmitt *et al*., 2002; Sumara *et al*., 2004; Van Vugt *et al*., 2004). These data suggested a dual role for Plk1 in the recruitment and activation of target proteins required for centrosome maturation and bipolar spindle formation.

One of the most intriguing features of Plk1 are the dynamic changes of localization and kinase activity during mitosis. Localization of Plk1 to the centrosome persists from early mitosis until late anaphase (Shirayama *et al*., 1998; Bahler *et al*., 1998; Logarinho and Sunkel, 1998; Moutinho-Santos *et al*., 1999; Mulvihill *et al*., 1999). Expression of a recombinant Green Fluorescent Protein (GFP) has confirmed the kinetochore/centromere localization of Plk1 and suggested that Plk1 moves to the kinetochore to possibly regulate chromosome

and chromatin separation during anaphase (Arnaud *et al*., 1998). Subsequently, Plk1 is located in the central spindle and the midbody region (Shirayama *et al*., 1998; Bahler *et al*., 1998; Logarinho and Sunkel, 1998; Moutinho-Santos *et al*., 1999; Song *et al*., 2000).

In mammalian cells, the C-terminus of Plk1 alone directs to centrosomes and midbody (Seong *et al*., 2002; Jang *et al*., 2002a). Alignment of the C-terminal regions of several polo-related kinases revealed that there is a motif with significant homology (polo-box) (Hudson *et al*., 2001), and that the introduction of a mutation in the polo-box disrupted the subcellular localization of Plk1 (Lee *et al*., 1998). Mutations in the polo-box of Cdc5 (the yeast homolog of Plk1), that do not affect the kinase activity, abolish the ability for localization to the mitotic apparatus in budding yeast (Lee *et al*., 1998; Song *et al*., 2000). Plo1 (a Plk1 homolog in fission yeast) interacts with multiple proteins, including cell cycle regulators, in a polo-box-dependent manner (Reynolds and Ohkura, 2003). These data, therefore, suggested that the polo-box is an essential motif for biological activity and the targeting of Plk in mitosis.

Despite a growing list of physiological substrates for Plk1, such as Cdc25, Myt1, cyclin B, NudC, cohesion, and TCTP that have been uncovered recently, the mechanism through which Plk1 regulates bipolar spindle formation, centrosome maturation, or cytokinesis remains unclear (Toyoshima-Morimoto *et al*., 2002; Sumara *et al*., 2002; Yarm, 2002; Nakajima *et al*., 2003; Jackman *et al*., 2003; Zhou *et al*., 2003). To better understand various Plk1 functions, it is important to identify Plk1 target proteins and regulators. The results of this study demonstrate several Plk1-interacting proteins through use of the N-terminal and polo-box motif of Plk1 as baits in yeast two-hybrid screens. To avoid the mitotic defect and abnormality by overexpression of the C-terminal domain in cells, we constructed a core polo-box motif which is sufficient to localize target organelles, and used this motif as bait in yeast two-hybrid screens without evidence of cellular toxicities.

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. The standard calcium chloride technique of DNA transfection was carried out except for the substituted use of HEPES-buffered saline (HBS) (Chen and Okayama, 1987; Jang *et al*., 2002a).

Preparation of plasmids. The polo-box motif (residue 400 to 454) and C-terminal domain (residue 305 to 603) were amplified by PCR from pCMV-FLAG-Plk (wild-type) (Jang *et al*., 2002a). The N-terminal domain of Plk1 was subcloned into various vectors from pCMV-FLAG-Plk (wild-type) by using SalI and BamHI. The DNA fragments were cloned into pLexA (Jang *et al*., 2004) for a yeast two-hybrid screen, and into pGEX-4T-2 for the expression of proteins in *E. coli*. For subcellular localization, they were cloned into the pEGFP-C1^{TM} (Clontech) vector. All constructs were confirmed by DNA sequencing.

Yeast two-hybrid screening. The N-terminus, C-terminus, and polo-box motifs were cloned into the pLexA in frame with the LexA DNA-binding domain. A yeast strain (EGY48) carrying a reporter gene (*LEU2*) was cotransformed with a human HeLa cDNA library (clarify, you do not mean the whole library) fused to *E. coli* acid blob domain B42 and the bait plasmids, pLexA-2xpolo (tandem repeat of amino acid 400-405), pLexA-Plk-N (amino acid 1-401), or pLexA-Plk-C (amino acid 305-603). Transformation was carried out by using the lithium acetate method (Gietz *et al*., 1992). Leucine-positive colonies were identified by a filter-lifting assay for β-galactosidase activity. Library-derived DNA was prepared from candidate clones and then analyzed 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 their binding to amylose resins (New England Biolab). Amylose resins were incubated with cell extracts which contained the GST fusion proteins. After the pull-down of beads, the bound GST proteins were detected by using anti-GST antibodies. Immunoprecipitation was achieved by lysing the cells 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 a protease inhibitor mixture. To serve as substrates for the kinase assay, the immunoprecipitates were washed once 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 analyzed (Jang *et al*., 2002b).

Immunofluorescence and microscopy. HeLa cells transfected with pEGFP constructs were grown on acid-treated coverslips, that were coated with a fibronectin solution (Sigma, F1141), and fixed with 3% paraformaldehyde/methanol (Jang *et al*., 2002a). Coverslips were washed three times in PBS, and used for multiple experiments. DNA in the cells was detected using propidium iodide. Following three final washes with PBS, the signals on the coverslips were detected with a Zeiss LSM510 confocal microscope.

Results and Discussion

N-terminal construct of Plk1: The N-terminal domain of mammalian Plk1 is localized in the cytosol. To investigate Plk1 functions in cells, we focused on the detection of cellular target proteins of Plk1. We initially separated the total Plk1 (amino acid 1-603, Plk in Fig. 1) into two parts: an N-terminal domain (amino acid 1-401, Plk-N in Fig. 1) and a C-terminal domain (amino acid 305-603, Plk-C in Fig. 1). The N-terminal domain of Plk1 has a conserved catalytic domain for protein kinase activity. Although the N-terminal domain dictates enzyme catalysis, it has been reported that the N-terminal

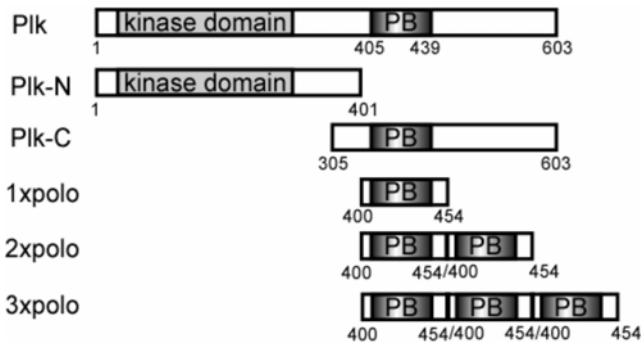


Fig. 1. Construction of the Plk1 domains. The full length of Plk1 (Plk) is 603 amino acids and contains a kinase domain and a polo-box (PB). Actually, two predicted polo-boxes in the C-terminal region of Plk1 (residue 405-439 and 506-598), but we didn't mark the second one in this cartoon. The N-terminal domain (residue 1-401) and the C-terminal domain (residue 305-603) were constructed for screening Plk1-interacting proteins (Plk-N & Plk-C). The 1xpolo indicates a construct containing the core segment of the polo-box (residue 400-454). The tandem repeated constructs of 1xpolo are indicated as 2xpolo and 3xpolo.

domain of Plk1 possibly interacts with several other proteins, such as the tubulins (Feng *et al*., 1999) and the molecular chaperones (Simizu and Osada, 2000), suggesting that the Plk kinase domain is capable of forming stable associations with target proteins. Therefore, we constructed an expression plasmid for the N-terminal domain of Plk1 (amino acid 1-401) to screen potential Plk1-interacting proteins (Fig. 1, Plk-N). To detect the localization of the N-terminal domain, we fused the domain with Green Fluorescence Protein (GFP) and allowed expression in HeLa cells. Interestingly, this domain protein expression was totally eliminated from the nucleus and was dispersed into the cytosol during interphase (Fig. 2A, upper panels). Even in the mitotic cells, the N-terminal domain of Plk1 was seen to be excluded from the chromosomal DNA region (Fig. 2A, lower panels).

C-terminal construct of Plk1: The core polo-box motif of mammalian Plk1 mediates its subcellular localization. Multiple roles for Plk1 during mitosis and cytokinesis are supported by the dynamic pattern of its localization.The Plk1 is mainly localized at the centrosomes in interphasic cells. As mitosis progresses, a fraction of Plk1 is redistributed to the microspindle and kinetochore, while the remaining fraction is at the spindle poles. During later stages, Plk1 concentrates in the midbody region (Jang *et al*., 2002a). Because the subcellular localization of Plk1 is mediated by the C-terminal domain, it is possible that Plk1 interacts with proteins in the centrosomes, kinetochore, and midbody through its C-terminal domain. In our initial Plk1-target screening, we used

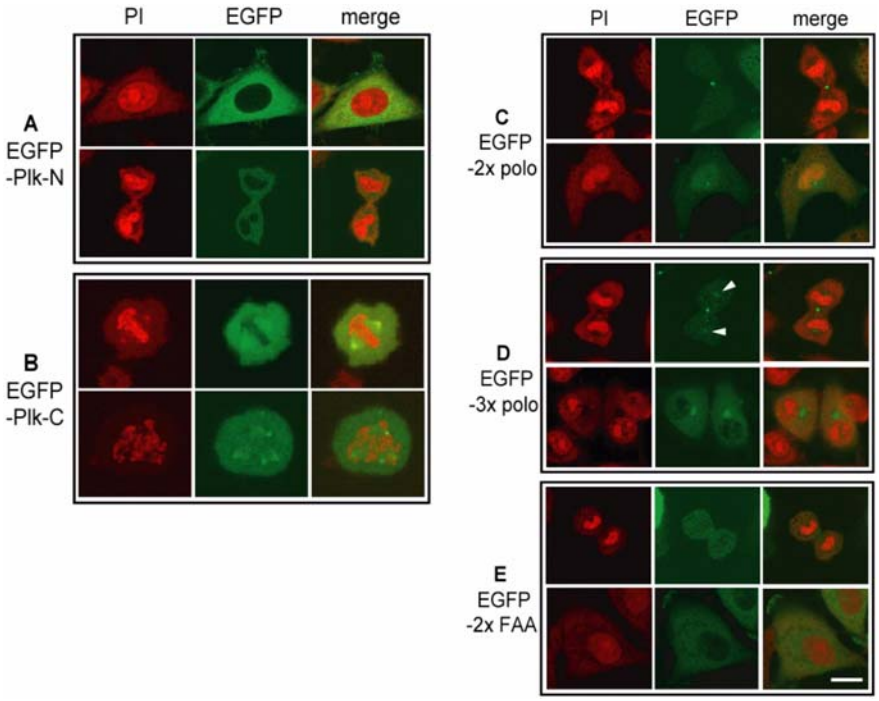


Fig. 2. Subcellular localization of the domain constructs for Plk1 and the polo-box motifs. HeLa cells were transfected with pEGFP-Plk-N (A), pEGFP-Plk-C (B), pEGFP-2xpolo (C), and pEGFP-3xpolo (D). As a negative control, three point mutations (W414F, V415A, and L427A) were introduced in 2xpolo, and the mutant construct (pEGFP-2xFAA) was then transfected into HeLa cells (E). Transfectants were permiabilized, fixed, and stained with propidium iodide (PI) for DNA as described in the Materials and Methods. Note that EGFP-FAA does not indicate a specific signal in the centrosome, midbody region, or kinetochores. Arrowheads point to the position of the kinetochores. The scale bar represents 10 mm.

299 amino acids of the C-terminal domain (amino acid 305-603) construct for bait (Fig. 1, Plk-C). Although this construct could be clearly localized in the centrosomes, most of the cells that overexpressed this domain had abnormal centrosomes in their positions and numbers (Fig. 2B, lower panels). A cellular defect based on C-terminus expression is likely caused by inhibition of endogenous Plk1 through a direct interaction of the Plk1 and the C-terminal domain (Jang *et al*., 2002a). Because of this mitotic abnormality, the C-terminal construct of 299 amino acids needed to be modified. Although there have been many efforts to identify Plk1-target proteins by using the C-terminal domain, few proteins have been reported by the two-hybrid technique, which suggested there could be difficulties in using the C-terminal or full length Plk1. There is a conserved motif in the C-terminal region of Plk1, however, named 'Polo-box', which is important for Plk1 localization. Therefore, we shortened the length of bait in the C-terminal domain into 54 amino acids, which contained the core sequence of the polo-box. When a 54 residue-length (amino acid 400-454) of this motif (Fig. 1, 1xpolo) was fused with GFP and applied for localization, we were able to detect their weak localization in the centrosomes (data not shown). To increase the likelihood of localization, we constructed tandem repeats of this fragment (Fig. 1, 2xpolo & 3xpolo). Interestingly, tandem repeats clearly localized into centrosomes in both mitotic and interphasic cells (Fig. 2C & D). In addition to the centrosomes, they also concentrated in the midbody region. Moreover, by using the tandem repeats of the polo core motif, we detected the kinetochore region, which is a structure for chromosome segregation that also contains the anaphase promoting complex (Fig. 2D). These data suggested that the core of the polo-box motif is sufficient to localize to Plk1 target organelles. When several point mutations were introduced into this motif (e.g. W414F/V415A/L427A), the subcellular localization of the polo-box motif was undetectable (Fig. 2E). These findings coincide with previous reports that a Plk1 containing a frame shift mutation in the polo-box did not localize properly (Lee *et al*., 1998). Thus, these tandem repeat polo-box constructs were used for target protein screening and *in vitro* binding experiments.

Yeast two-hybrid screening for Plk1-target proteins by using the N-terminus and polo-box motif. The N-terminal domain of Plk1 (Plk-N) and a tandem repeat of the polo-box motif (2xpolo) were cloned into the pLexA vector following the LexA protein coding region. The cDNAs from HeLa cells were ligated into the pJG4-5 vector and fused with B42 protein into the N-terminus. Two plasmid constructs were introduced into yeast strain EGY48, which has two reporters: *LEU2* and LacZ. These reporter genes were regulated by the LexA-B42 protein complex. In the first round of screening, the colonies grown as Leu^+, His^+, and Trp^+ were selected: 220 positive colonies from the screening by 2xpolo and 125 positive colonies from the screening by Plk-N. For the second-round screening, the positive colonies from the 1st screening were applied to a β-galactosidase filter assay. Finally, 71 of the 220 colonies and 48 of the 125 colonies from the screening by 2xpolo and Plk-N were shown as strong blue signals in the filter assay. The plasmids containing cDNA fragments were isolated from these positive colonies and characterized by sequencing. As expected, there were many false positives. In the results of the 2xpolo-screening, (71 cases) 12 were ribosomal proteins (16.9%), and 16 were metabolic enzymes such as enolase and dehydrogenase (22.5%). Similarly, in the Plk-N-screening (48 cases), 11 were ribosomal proteins (22.9%), and 9 were metabolic enzymes (18.8%). The elongation factor-1 (EF-1) was a protein frequently captured as a false-positive, and this protein was also screened as 5.6% (4 of 71) and 4.2% (2 of 48) of the total cases from the 2xpolo and Plk-N screenings, respectively. Interestingly, α- (1 of 71) and β-tubulins (3 of 71) interacted with the polo-box motif of Plk1 (data not shown). Previously, Feng *et al*. (1999) reported that the tubulins associated with both the N- and C-terminal domains of Plk1 *in vitro*. Our data coincided with the notion that the polo-box is the essential motif for the interaction of tubulins with the C-terminal domain of Plk1, however, we were unable to verify tubulin clones in the Plk-N screening.

The representative positive clones in the Plk-N and 2xpolo screening are shown in Tables 1-1 and 1-2. The clones that contained the DNA fragment of the same genes are selected in this screening. To investigate whether their interactions were dependent on vectors, cDNA fragments of the positive clones were transferred to the pLexA vector from pJG4-5, and both the Plk-N and 2xpolo were cloned into the pYesTrp vector that contained the B42 domain. The interactions between clones were analyzed by both liquid and filter assays for β-galactosidase activity. Although the vectors differed, cDNA from positive clones interacted with Plk-N- or 2xpolo constructs, and their associations were over 40 times stronger than the negative control, suggesting that these proteins interact with Plk-N or 2xpolo independent of the vectors (Table 2).

To investigate whether these interactions were domain-specific, two baits were exchanged with each other, and their cross binding activity analyzed. While the PPIA and CCT clones were screened out as Plk-N interacting clones, they nonetheless interacted with the polo-box motif (Table 3). Conversely, there was no cross activity in the polo-box interacting clones.

An *in vitro* protein interaction experiment was conducted that also supported their interaction. Two baits and cDNA fragments of positive clones were transferred to *E. coli* expression vectors, pGEX and pMAL, respectively. After induction by treatment with IPTG, the MBP-fusion proteins were purified from cell extracts by using MBP-beads. After induction of the GST-fusion baits, cell extracts were prepared and incubated with the purified MBP-fusion proteins. GST antibodies were used to detect baits associated with MBP-proteins. Four representative MBP-proteins were interacted with both 2xpolo and 3xpolo as shown in Fig. 3A. The

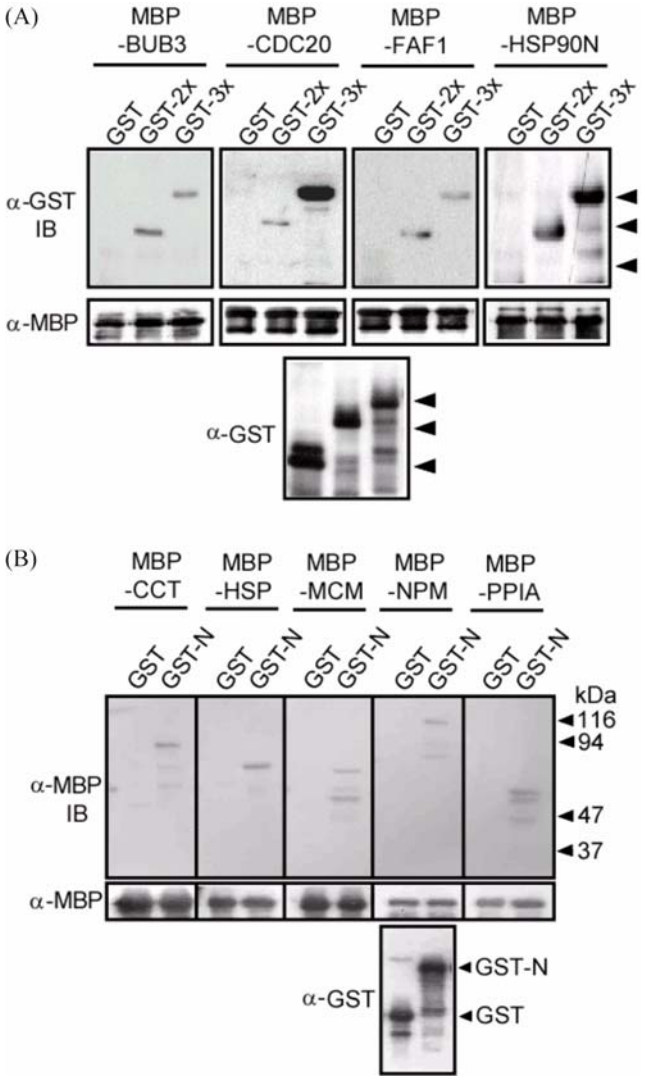


Fig. 3. N-terminal domain and polo-box interactions with proteins as characterized ***in vitro.*** The proteins from the positive clones were fused with MBP, and both polo-boxes (A) and the N-terminus of Plk1 (B) were fused with GST. (A) Cell extracts of GST-polo-boxes were incubated with the MBP-purified proteins and the pull-downs were probed by Western blotting with anti-GST antibodies (upper panel, α-GST IB). The used amounts of MBP-fusion protein purified and GST-baits in cell extracts were estimated by anti-MBP and anti-GST antibodies, respectively (middle and lower panels). (B) Cell extracts of MBP-fusion clones were incubated with the GST-purified Plk-N and the pull-downs were probed by Western blotting with anti-MBP antibodies (upper panel, α-MBP IB). The used amounts of MBP-proteins in cell extracts and GST-Plk-N purified were estimated by anti-MBP and anti-GST antibodies, respectively (middle and lower panels).

interactions between both MBP-Cdc20 or -Hsp90N and GST-3xpolo were stronger than identified for GST-2xpolo (Fig. 3A), which suggested that the three copy tandem repeats of the polo-box has a stronger affinity on the target proteins than

Table 1-1. Representative positive clones interacted with the N-terminus of Plk1. After DNA sequencing, the length of insert cDNA fragments was indicated as 'clone length'. Some clones were characterized as full length ORF (PPIA, protein kinase C inhibitor-1, and nucleophosmin).

Table 1-1 N-term-interacting clones	full length (total amico acids)	clone length* (start - end)
Peptidylpropyl isomerase A (PPIA)	165 amino acids	1 - 165
Minichromosome maintenance protein 3 (MCM3)	808 amino acids	682 - 808
Protein kinase C inhibitor 1	126 amino acids	1 - 126
Chaperonin CCT	539 amino acids	105 - 539
putative cell cycle control protein	527 amino acids	1 - 500
Heat shock protein 90	724 amino acids	501 - 724
Nucleophosmin	294 amino acids	1 - 294

Table 1-2. Representative positive clones interacted with 2xpolo. After DNA sequencing, the length of insert cDNA fragments was indicated as 'clone length'.

Table 1-2 Polo Box-interacting clones	full length (total amico acids)	clone length* (start - end)
Cell cycle protein CDC20	609 amino acids	110 - 609
RanGTPase	243 amino acids	46 - 243
Budding uninhibited by benzimidazoles 3 (BUB3)	328 amino acids	1 - 162
Fas-Associated factor 1 (FAF1)	650 amino acids	448 - 650
BCL2 homolog MCL1	350 amino acids	186 - 350
α-catenin	906 amino acids	737 - 906
Heat shock protein 90	724 amino acids	1 - 258
HsCdc18	560 amino acids	202 - 560

the two copy repeat constructs. Interactions between five representative Plk-N-interacting proteins and GST-Plk-N were shown *in vitro* (Fig. 3B). After induction of five MBP-fusion proteins, cell extracts were prepared and incubated with the purified GST-fusion baits. MBP antibodies were used to detect baits associated with GST-Plk-N. CCT (chaperones) containing TCP-1, 110 residue to the end), C-terminal of HSP90 (500 residue to the end), MCM3 (DNA replication licensing factor, 633 residue to the end), and full lengths of nucleophosmin and PPIA (peptidyl-prolyl cis-trans isomerase A), which consisted of the same constructs with two-hybrid clones, were provided for interaction with GST-Plk-N *in vitro*. Other MBP-fusion proteins were also interacted with GST fusion bait (data not shown).

Interestingly, both the N-terminal and the C-terminal domains of Hsp90 interacted with Plk1. The C-terminal domain of Hsp90 associated with the N-terminal domain of Plk1,

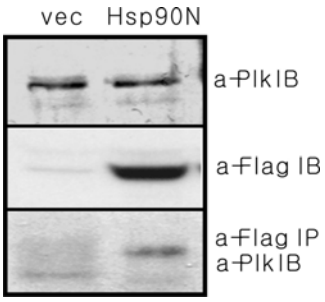


Fig. 4. Endogenous Plk1 interacts with Hsp90N in mitotic HeLa cells ***in vivo.*** HeLa cells were transfected with FLAG-tagged Hsp90N (amino acid 1-260) (Hsp90N) and vector only (vec). After 24 h in transfection, nocodazole was added for mitotic arrest for a 16 h period and the transfectants collected. FLAG-tagged proteins were immunoprecipitated by anti-FLAG antibodies. To detect endogenous Plk1 interacted with Hsp90N proteins, immunoprecipitates were probed by Western blotting with anti-Plk1 antibodies (lower panel, α-FLAG IP/α-Plk IB). The endogenous Plk1 and FLAG-Hsp90N in mitotic HeLa cell extracts were estimated by anti-Plk1 antibody and anti-FLAG antibody, respectively (upper and middle panels).

whereas the N-terminal half of Hsp90 associated with the polo-box motif (Table 1). Previously, Simizu *et al*. (2000) had reported that Hsp90 is a target protein of Plk1 in mammalian cells and had characterized the Hsp90-interacting domains of Plk1. They suggested that molecular chaperones could be targeted to Plk1 and could regulate Plk1 stability. As expected, endogenous Plk1 interacted with the N-terminus of Hsp90 (amino acid 1-260) in mitotic HeLa cells (Fig. 4). In this regard, Liu *et al*. (2005) reported recently that the molecular chaperone protein (CCT) is necessary for Plk1 maturation. In our results, CCT as well as Hsp90, was characterized as one of the target proteins of Plk1, thereby indicating that the tandem repeat construct of the polo box is an excellent bait for interacting with cellular target proteins.

Plk1 localizes in various cell organelles during mitosis such as centrosome, kinetochore, and the midbody region. As shown in Fig. 2, the tandem repeats of the polo-box (2xpolo and 3xpolo) concentrated in these organelles. According to subcellular localization, we had predicted that the tandem repeat motif would be useful for the screening of target proteins, components of these organelles. Indeed, RanGTPase, Cdc20 and Bub3 were characterized as polo-box-interacting proteins (Table 1-2). These data strongly suggest direct evidence for Plk1 to localize in centrosomes and the kinetochore and thereby perhaps regulate these components. Recently, we reported that an association between Plk1 and Ran protein occurred in the mitotic centrosome region (Jang *et al*., 2004). In addition to the centrosome, Plk1 might localize in the kinetochore region through interactions with Cdc20 and Bub3. The regulation of these proteins by Plk1 in centrosome maturation or chromosome segregation is planned for further study.

Table 2. Confirmation of positive clones by the exchange of vectors. The DNA fragments of bait were cloned into the pJG4-5 vector, which were used for cDNA expression, and the inserts of positive clones were transferred into the pLexA vector. The indicated plasmids were transfected into the yeast strain (EGY48) and individual cotransformants were patched onto SD/gal/raf/-His/-Trp/-Leu/-Ura to select for all plasmids expressing interactive hybrid proteins (see Materials and Methods), β-gal lift assays were performed (β-gal filter assay). The blue color indicates a positive signal in the assay. For quantitative analysis of protein interactions, colonies were subjected to a liquid β-galactosidase assay as described previously (relative β-gal activity). The N-terminal domain- and 2xpolo-interacting clones are indicated in (A) and (B), respectively. The intensity of blue color was indicated as '+'; a negative signal on the β-gal lift assay was indicated as '-'.

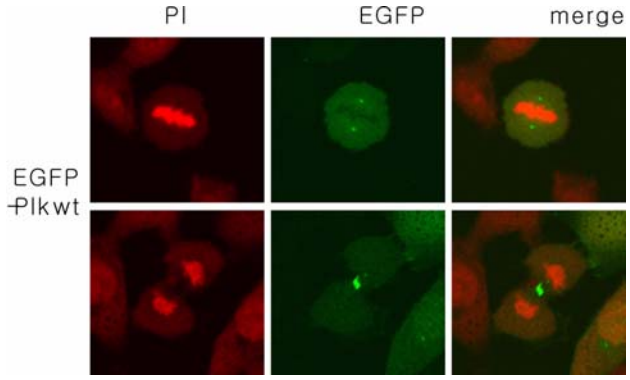
A Bait : N-terminus (1-401)	relative β-gal activity	β-gal filter assay
pYes + pLex	1.00	-
pYes-Plk-N + pLex	1.12	-
pYes-Plk-N + pLex-PPIA	40.23	+
pYes-Plk-N + pLex-MCM3	39.00	+
pYes-Plk-N + pLex-PKCI1	42.66	+
pYes-Plk-N + pLex-CCT	39.35	+
pYes-Plk-N + pLex-CCCP	32.00	+
pYes-Plk-N + pLex-HSP90C	39.00	+
pYes-Plk-N + pLex-NPM	42.05	+

B Bait : 2xPolo-Box (400-454/400-454)	relative β-gal activity	β-gal filter assay
pYes + pLex	1.00	-
pYes-Polo + pLex	0.95	-
pYes-Polo + pLex-CDC20	42.66	+
pYes-Polo + pLex-Ran	35.00	+
pYes-Polo + pLex-BUB3	57.23	+
pYes-Polo + pLex-FAF1	32.35	+
pYes-Polo + pLex-MCL1	32.00	+
pYes-Polo + pLex-α-catenin	39.00	+
pYes-Polo + pLex-HSP90N	41.05	+
pYes-Polo + pLex-hCDC18	34.05	+

A group of the identified target proteins were apoptosis-related, which interacted with the polo-box motif (Table 1-2, FAF1 and MCL1). To date, there have been several reports regarding Plk1 function in apoptosis. Thus, when Plk1 was silenced in cancer cells by small interference RNA (SiRNA), the cells ceased to proliferate and apoptosis was induced (Liu and Erikson, 2003). Because Plk1 is a key factor for progression of mitosis, it was predicted that cell proliferation would be inhibited by Plk1 silencing. We have in addition provided evidence that Plk1 may regulate the apoptosis pathway by a direct interaction with several apoptotic factors.

Table 3. Confirmation of domain specificity of the positive clones. The pJG4-5 constructs of the N-terminal domain-interacting proteins were applied to two-hybrid by using pLexA-2xpolo as bait. Plasmid constructs of polo-box-interacting proteins were applied to two-hybrid by using pLexA-Plk-N. Individual cotransformants were patched and β-gal lift assays were performed. '+' indicates a positive blue signal. '-' indicates a negative signal. PPIA and CCT were interacted with both the Plk-N and 2xpolo components.

Clones	Bait	
	N-term	2xpolo
Peptidylpropyl isomerase A (PPIA)	+	+
Minichromosome maintenance protein 3 (MCM3)	+	-
Protein kinase C inhibitor 1	+	-
Chaperonin CCT	+	+
Putative cell cycle control protein	+	-
Heat shock protein 90 C	+	-
Nucleophosmin	+	-
Cell cycle protein CDC20	-	+
RanGTPase	-	+
Budding uninhibited by benzimidazoles 3 (BUB3)	-	+
Fas-Associated factor 1 (FAF1)	-	+
BCL2 homolog MCL1	-	+
α-catenin	-	+
Heat shock protein 90 N	-	+
HsCdc18	-	+



Supplementary data-1. Subcellular localization of full length of Plk1.

In conclusion, we have identified two subjects which are important for the study of intracellular functions of Plk1. First, we have constructed a centrosome/kinetochore-specific targeting peptide with applications to the screening of cellular components regulated by Plk1. Importantly, this peptide is no effect on cell growth, whereas the C-terminal domain of Plk1 induces mitotic chaos. Therefore, this specific motif may be useful as a cellular marker of the centrosome/kinetochore as well as the midbody region and without toxic effects. Secondly, we identified several possible target proteins of Plk1 by yeast-two hybrid screening. Various targets screened by using the N-terminal and polo-box motif as baits provide potential pathways for the further study of Plk1 functions within cells.

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