Polo boxes form a single functional domain that mediates interactions with multiple proteins in fission yeast polo kinase

Nicola Reynolds* and Hiroyuki Ohkura‡

The Wellcome Trust Centre for Cell Biology, Institute of Cell and Molecular Biology, The University of Edinburgh, Edinburgh EH9 3JR, UK
*Present address: MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, UK
‡Author for correspondence (e-mail: h.ohkura@ed.ac.uk)

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Summary

Polo kinases play multiple roles in cell cycle regulation in eukaryotic cells. In addition to the kinase domain, conservation at the primary sequence level is also found in the non-catalytic region mainly in three blocks, namely the polo boxes. Although several studies have implicated the polo boxes in protein localisation, no systematic study to elucidate the roles of individual polo boxes has been carried out. Here we show, by using fission yeast, that the polo boxes form a single functional unit that is essential for both cellular function and cell-cycle-regulated localisation to the spindle pole bodies. Various polo box mutations abolish the mitotic arrest seen upon overexpression of *plo1* but do not prevent the untimely septation seen under these conditions, showing that the functions of Plo1 may be separated. Plo1 interacts with multiple proteins including cell cycle regulators in a polo-box-dependent manner. Isolation of mutants that differentially disrupt these interactions revealed a role for the polo boxes in mediating protein-protein interactions.

Key words: Polo, Kinase, Cell cycle, Fission yeast

Introduction

The cell division cycle in eukaryotic cells is a highly regulated process requiring a range of events to be carried out sequentially. The polo-like kinases (or polo kinases) are a family of protein kinases that are implicated in numerous events throughout mitosis (reviewed by Glover et al., 1996; Lane and Nigg, 1997; Glover et al., 1998; Donaldson et al., 2001). Although mammalian cells contain more than one polo-like kinase, yeasts and *Drosophila* have only one. An understanding of how polo kinases regulate mitotic events will be essential to understanding molecular mechanisms of the mitotic progression as a whole.

One of the most intriguing aspects of polo kinase function is the variety of tasks they execute throughout the cell cycle. Polo kinases are required at several key points through mitosis, starting from control of the G2/M transition through phosphorylation of Cdc25C and mitotic cyclins (Abrieu et al., 1998; Karaiskou et al., 1999; Kumagai and Dunphy, 1996; Ouyang et al., 1997; Qian et al., 1998; Toyoshima-Morimoto et al., 2001) and a role in the DNA damage checkpoint to prevent entry into mitosis (Sanchez et al., 1999; Smits et al., 2000; Toczyski et al., 1997). At the beginning of mitosis, various proteins are recruited to the centrosomes, a maturation process which requires polo kinases (Sunkel and Glover, 1988; Lane and Nigg, 1996). Polo kinases are also required for the establishment of a bipolar spindle (Ohkura et al., 1995; Lane and Nigg, 1996; Qian et al., 1998), a conserved function which is evident from the phenotype of the original *Drosophila polo*[1] mutant (Llamazares et al., 1991; Sunkel and Glover, 1988).

Equally, polo kinases are important for exit from mitosis. A role in the metaphase to anaphase transition via an interaction with the anaphase promoting complex/cyclosome (APC/C) has been demonstrated (May et al., 2002; Descombes and Nigg, 1998; Charles et al., 1998; Shirayama et al., 1998). In addition, budding yeast polo kinase phosphorylates cohesin to allow proteolysis by separase in order to initiate anaphase (Alexandru et al., 2001).

Fission yeast polo kinase, Plo1, is required for formation and correct positioning of the septum and overexpression induces septation even in interphase cells (Ohkura et al., 1995; Bahler et al., 1998). Overproduction of murine or budding yeast polo kinases in budding yeast cells also induces septation in a non-catalytic domain dependent manner (Lee and Erikson, 1997; Song et al., 2000), and a physical interaction has been demonstrated between the budding yeast polo kinase, Cdc5p, and septins (Song and Lee, 2001). This function in cytokinesis has also been shown to be conserved in *Drosophila* (Carmena et al., 1998; Herrmann et al., 1998).

Dynamic, cell cycle regulated localisation and kinase activation during mitosis is a feature common to all members of the family. Localisation to the SPB/centrosome occurs early in mitosis and persists until late anaphase, when the protein has been seen to relocalise to the midbody or to the future site of cell cleavage (Bahler et al., 1998; Golsteyn et al., 1995; Logarinho and Sunkel, 1998; Moutinho-Santos et al., 1999; Mulvihill et al., 1999; Shirayama et al., 1998; Song et al., 2000). Detailed study in fission yeast has indicated that localisation to the SPB takes place very early in mitosis, prior to full activation of catalytic activity (Tanaka et al., 2001). This cell cycle regulated catalytic activation and localisation is

likely to play an important role in the execution of polo kinase function.

Polo-like kinases are characterised by an amino terminal catalytic domain, and a carboxy terminal non-catalytic domain consisting of three blocks of conserved sequences known as polo boxes (Glover et al., 1996) (Fig. 1). Studies have been carried out to identify the role of this non-catalytic domain in budding yeast and mammalian cultured cells. In mammalian cells, the C-terminus of mammalian Plk1 alone directs localisation to centrosomes (Jang et al., 2002; Seong et al., 2002). A mutation in polo box 1 abolished the ability of mammalian or budding yeast polo kinase to localise to the mitotic apparatus in budding yeast (Lee et al., 1998; Song et al., 2000) or to complement a budding yeast mutant (Jang et al., 2002; Lee et al., 1998; Song et al., 2000). Also, the effects of overexpression of the kinase in budding yeast or mammalian cells were shown to be dependent on polo boxes (Lee et al., 1998; Seong et al., 2002). However, whether the non-catalytic region consists of functionally separable sub-domains, in particular in the light of multi-functional nature of polo kinase, has not been addressed.

Fission yeast is an excellent model organism for the dissection of the multi-functional nature of polo kinase due to the ease with which it is genetically manipulated and the clarity with which multiple functions are observed. In fission yeast, deletion of the polo kinase gene, *plo1*$^+$, results in three major defects – failure to establish spindle bipolarity, failure to form septa or misplacement of septa (Ohkura et al., 1995; Bahler et al., 1998). In addition, overexpression of the polo kinase results in two clear phenotypes, cells displaying multiple septa and mitotically arrested cells with monopolar spindles (Ohkura et al., 1995). Involvement in entry into mitosis has been suggested through the interaction with the SPB protein Cut12 and the NimA-like kinase Fin1 (Mulvihill et al., 1999; Grallert and Hagan, 2002) and a role in metaphase to anaphase transition via an interaction with the APC/C subunit Cut23 has also been demonstrated for fission yeast Plo1 (May et al., 2002). Furthermore, it has been shown that Plo1 is at the top of the septum initiating network (SIN) (Tanaka et al., 2001) demonstrating that many of the conserved functions of polo kinases may be studied using fission yeast as a model organism.

In this study we examined the roles of conserved amino acid sequences in the fission yeast polo-like kinase (Plo1) and in particular their relationship with multiple functions. Functions were explored by overexpression experiments and by stable expression of mutant genes at wild-type levels in a *plo1* deletion background. We have shown, using a combination of site directed and a novel random mutagenesis method, that the polo boxes form a single functional unit required for in vivo function, SPB localisation and multiple protein-protein interactions.

Materials and Methods

Molecular techniques

General molecular techniques were carried out according to Sambrook et al. (Sambrook et al., 1989). Site directed mutagenesis of *plo1* was carried out in pTZ19U (Biorad) by using the Muta-Gene Phagemid in vitro mutagenesis kit (Biorad) according to the manufacturer's instructions. Mutations were confirmed by sequencing of the *plo1* gene.

Fission yeast techniques

Fission yeast analyses were carried out as described (Moreno et al., 1991; Ohkura et al., 1995). Strains used in this study are shown in Table 1.

In overexpression experiments, *plo1* mutants were expressed from the *nmt1* promoter in pREP1 (Maundrell, 1993). Cells were grown in the presence of thiamine and then washed in sterile water before growth in two parallel cultures, either in the presence or absence of 4 μM thiamine, for 15-16 hours.

For plasmid shuffling experiments, spores were prepared from a diploid strain heterozygous for deletion of *plo1* (Sp269) carrying wild-type *plo1* on a *ura4*$^+$ marked plasmid [pUR*plo1*$^+$ (May et al., 2002)] and mutant *plo1* in pREP1. Germination was carried out in the presence of thiamine and *plo1Δ* haploids selected. The presence of pUR*plo1*$^+$ maintains viability of the deletion while thiamine represses expression of the *plo1* mutants. The strains were replica plated three times to media containing uracil (both in the presence and absence of thiamine) to allow loss of the pUR*plo1*$^+$ plasmid. Cells that had lost the plasmid were selected by using 5-FOA, which kills Ura$^+$ cells. Cells were spotted on selective media in the presence or absence of both 5-FOA and thiamine. Abilities of each *plo1* mutant to complement a *plo1* deletion were tested either at approximately wild-type levels of expression (+ thiamine, *nmt1* repressed) or when overexpressed (– thiamine, ~100 times that of wild-type expression levels, *nmt1* de-repressed).

Spore germination analysis was carried out to determine whether expression of the mutant genes at wild-type level from an integrated copy were able to support growth in the absence of endogenous *plo1*$^+$. HA-tagged *plo1* mutants under the control of the attenuated *nmt1* promoter (derived from pREP41) were integrated at the *leu1* locus of a *plo1::his3*$^+$*/plo1*$^+$ diploid strain (Sp269). Sp269 was created by replacing the entire coding region of one *plo1* gene with *his3*$^+$ using a PCR-mediated method (Bahler et al., 1998). Integration was confirmed by PCR using primers 5′ to *leu1* and within the polo box domain of *plo1*. Comparison of the number of His$^+$ (*plo1Δ*) and His$^-$ (*plo1*$^+$) haploids obtained following spore germination revealed whether a particular mutant complemented the *plo1* disruptant. Full rescue of the deletion phenotype resulted in the ratio of His$^-$ to His$^+$ haploids of 1:1. Where no rescue occurred, no His$^+$ haploids were found.

Fluorescence microscopy

Samples were prepared and fixed as described previously (May et al., 2002; Ohkura et al., 1995). Localisation of the Plo1 mutant proteins in vivo was observed in strains expressing GFP-Plo1 under the control of the attenuated *nmt1* promoter by autofluorescence while Tat1 antibody (Woods et al., 1989) and Sad1 antibody (Hagan and Yanagida, 1995) were used for visualising microtubules and SPB, respectively. Cells were observed with an Axioplan 2 microscope (Zeiss). Images were captured using a CCD camera (Hamamatsu) and processed using Openlab2 (Improvision) and Photoshop (Adobe).

Two-hybrid analyses

Two-hybrid screening using *plo1*$^+$ as a bait was carried out as described (May et al., 2002) and positive interactors confirmed by isolation and reintroduction of the prey plasmids along with either pBTM116*plo1*$^+$ or pBTM116.

Isolation of *plo1* mutants, which retain only a subset of interactions was carried out as follows. For random mutagenesis of *plo1*, pBTM116*plo1*$^+$ was used as template in a PCR reaction using Taq polymerase without proofreading activity (Roche). Primers used in the reaction were complementary to the 5′ end of *plo1*$^+$ and to the *ADH1* terminator sequence, which is 3′ to the *plo1*$^+$ gene in pBTM116*plo1*$^+$. Strain L40 was transformed with gapped pBTM116 plasmid and PCR product. Gap repair in vivo resulted in the recreation of pBTM116*plo1*

Table 1. Strains used in this study

Strain	Genotype	Source
Sp159	*mad2::ura4$^+$ leu1-32 h*	(Kim et al., 1998)
Sp25	*975 h$^+$*	Ohkura Lab
Sp28	*leu1-32 ura4-D18 h$^-$*	Ohkura Lab
Sp33	*ura4-D18 h$^-$*	This study
Sp172	*ura4-D18 leu1::nmt41EGFPNplo1$^+$ ura4$^+$ h$^-$*	This study
Sp182	*ura4-D18 leu1::nmt41EGFPNplo1K69R ura4$^+$ h$^-$*	This study
Sp210	*ura4-D18 leu1::nmt41EGFPNplo1T197V ura4$^+$ h$^-$*	This study
Sp185	*ura4-D18 leu1::nmt41EGFPNplo1W497F ura4$^+$ h$^-$*	This study
Sp170	*ura4-D18 leu1::nmt41EGFPNplo1YQL508AAA ura4$^+$ h$^-$*	This study
Sp163	*ura4-D18 leu1::nmt41EGFPNplo1DHK625AAA ura4$^+$ h$^-$*	This study
Sp166	*ura4-D18 leu1::nmt41EGFPNplo1.1-633 ura4$^+$ h$^-$*	This study
Sp161	*ura4-D18 leu1::nmt41EGFPNplo1.1-583 ura4$^+$ h$^-$*	This study
Sp168	*ura4-D18 leu1::nmt41EGFPNplo1.1-533 ura4$^+$ h$^-$*	This study
Sp160	*ura4-D18 leu1::nmt41EGFPNplo1.1-483 ura4$^+$ h$^-$*	This study
Sp230	*ura4-D18 leu1::nmt41EGFPNplo1.313-684 ura4$^+$ h$^-$*	This study
Sp208	*ura4-D18 leu1::nmt41EGFPNplo1.472-684 ura4$^+$ h$^-$*	This study
Sp189	*ade6-M210 leu1-32 ura4-D18 h$^-$*	Ohkura Lab
Sp259	*leu1-32 his3-D1 h$^+$*	Sawin Lab
Sp260	*leu1-32 his3-D1 ura4-D18 ade6-M216 h$^-$*	Sawin Lab
Sp264	*his3-D1 ura4-D18 ade6-M210 leu1-32 h$^+$*	This study
Sp267	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1-32 h$^+$/h$^-$*	This study
Sp269	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1-32 plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp282	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1$^+$ ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp285	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1K69R ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp291	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1D181R ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp292	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1D181N ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp288	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/ leu1::nmt41 HA$_3$plo1T197V ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp289	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1L210W ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp290	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1P490L ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp283	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1YQL508AAA ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp284	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1DHK625AAA ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp296	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1.1-633 ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp299	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1.1-583 ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp297	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1.1-533 ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp295	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1.1-483 ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp310	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1K251E ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp307	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1S256P ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp308	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1V484A ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp313	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1V533A ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp312	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1L565F ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp309	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1F493L ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study
Sp311	*his3-D1/his3-D1 ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1::nmt41HA$_3$plo1R605P ura4$^+$ plo1$^+$/plo1::his3$^+$ h$^+$/h$^-$*	This study

with mutations in *plo1* (Muhlrad et al., 1992). Each L40 stain carrying mutagenised *plo1* bait constructs was mated with Y187 strains carrying prey plasmids. Mating efficiency was determined by growth on selective media and the two-hybrid interaction assessed by expression of the *HIS3* reporter gene (by growth on selective media lacking histidine and containing 10 mM 3-AT after 2 days at 32°C). Plasmids were isolated from strains with pBTM116*plo1* constructs of interest, re-transformed to confirm the interaction pattern, and sequenced to determine the mutation site.

Results

All three polo boxes are required for the interference of bipolar spindle formation, but none for induction of untimely septation upon overexpression of Plo1

Polo kinases have two conserved regions. One coincides with an amino-terminal catalytic (kinase) domain and the other is found in the carboxy-terminal non-catalytic region, mainly as three blocks called the polo boxes (Glover et al., 1996) (Fig. 1). In an attempt to define functional domains, we targeted highly conserved residues in both the catalytic and non-catalytic regions of the fission yeast polo kinase, Plo1, for site-directed mutagenesis (Fig. 1) and assayed functions of the mutant genes.

First the mutant genes were overexpressed in wild-type cells under the control of the *nmt1* promoter on a multicopy plasmid, pREP1. Overexpression of the wild-type *plo1* gene resulted in two major cell defects (Ohkura et al., 1995) (Fig. 2B). One is the induction of untimely septation, which can be seen in cells with multiple septa or mononucleated cells with septa. The other is a failure to establish the bipolarity of the mitotic spindle resulting in mitotic arrest, which can be seen as cells with overcondensed chromosomes and monopolar spindles.

To see whether the non-catalytic domain consists of functionally separable units, we tested various mutants in the polo boxes. These include various point mutations at highly conserved amino acids in polo box 1 (W497F, G505A, YQL508AAA and FN519AA), polo box 2 (L577A) or polo box 3 (DHK625AAA), and series of C-terminal deletions (50 amino acids each; 1-633, 1-583, 1-533, and 1-483). We found that all of the mutations created in the non-catalytic domain show identical effects upon overexpression. They all abolished the mitotic arrest phenotype but had little effect on induction of untimely septation (Fig. 2C,D,K as examples, and data not shown for other mutations). This demonstrates that the overexpression effects of *plo1$^+$* on spindle formation and

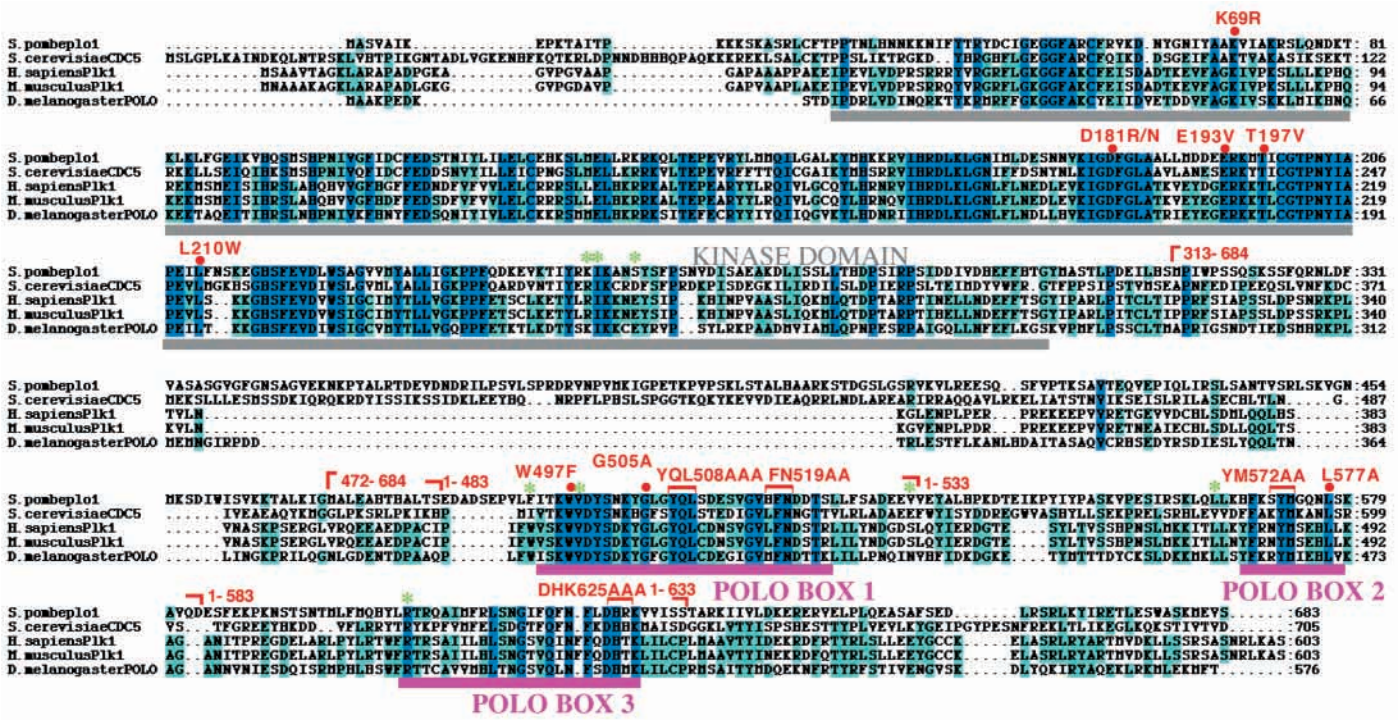

Fig. 1. Conservation of amino acid sequences among polo kinase homologues and mutations of *S. pombe* Plo1 created in this study. Residues shaded in dark or light blue represent identical residues among all or most of these polo kinase homologues respectively. Mutations or truncations created by site-directed mutagenesis are marked in red and sites of mutations created by random mutagenesis are marked in green.

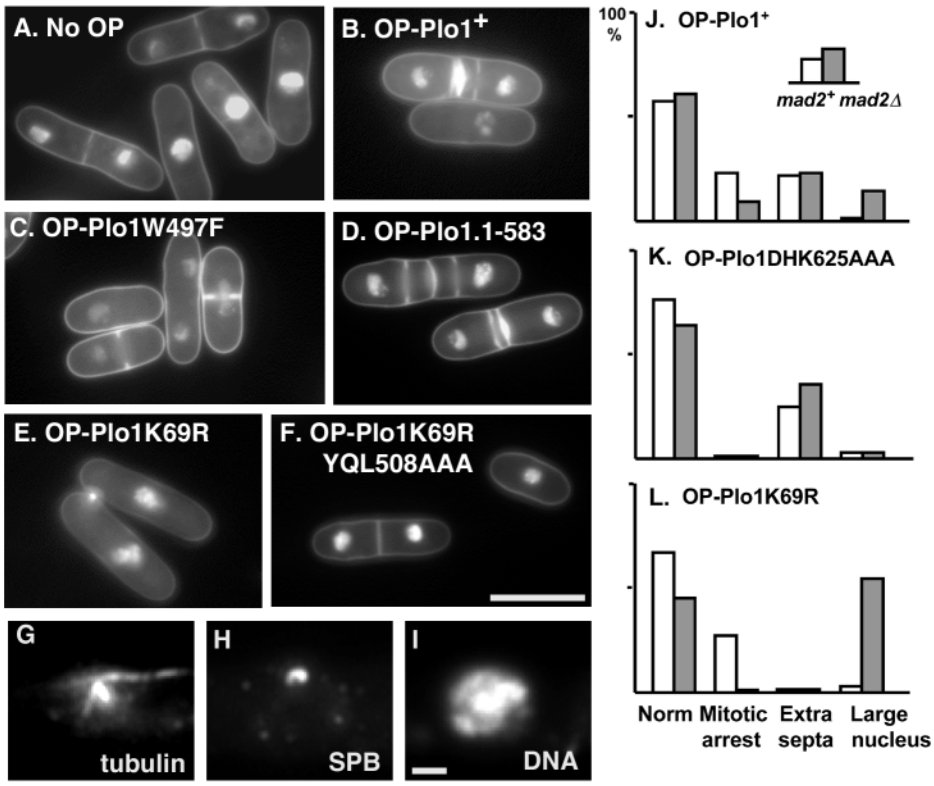

Fig. 2. Polo boxes are required for interference of bipolar spindle formation, but not for induction of septation upon overexpression of Plo1. Wild-type cells carrying *plo1* mutants in pREP1 under the control of the thiamine repressible *nmt1* promoter were grown in the absence of thiamine. The cells were fixed and stained with DAPI, which highlights DNA, cell outlines and septa. No defects were apparent with any of the plasmids in the presence of thiamine. Bar, 10 μm. (A) Empty vector, pREP1 has no effect on cell growth. (B) Overexpression of wild-type *plo1* (pREP1*plo1*$^{+}$) leads to mitotic arrest and cells with extra septa. (C,D) Overexpression of mutants in the polo boxes (*plo1W497F,plo1.1-583* are shown here as examples) resulted in uncontrolled formation of septa but no mitotically arrested cells. (E) Overexpression of a catalytic inactive *plo1* (*plo1K69R)* results only in mitotically arrested cells with highly overcondensed chromosomes with no cells forming extra septa. (F) Overexpression of a catalytically inactive polo-box mutant *plo1K69R YQL508AAA*. No abnormalities can be seen. (G-I) Monopolar spindles were associated with overcondensed chromosomes in cells overexpressing *plo1K69R*. The SPB component α-tubulin (G), Sad1 (H) and DNA (I). (J-L) Quantification of cytological defects seen upon overproduction of wild-type Plo1 (J), Plo1 with a polo box mutation (Plo1DHK625AAA; K) or kinase inactive Plo1 (Plo1K69R; L) in wild-type or *mad2* deletion strains. Cells were observed by DAPI staining after the removal of thiamine to induce expression from the *nmt1* promoter. Mitotic arrest caused by overproduction of inactive Plo1 is abolished by a mutation in the spindle checkpoint, *mad2Δ*.

septation are separable and that the former is dependent on the integrity of all of the polo boxes but the latter is not. Mutations in any one of the three polo boxes led to an identical effect suggesting that the polo boxes may act as a single functional unit.

On the other hand, mutations in the kinase domain of Plo1 result in the opposite effect. We tested various mutations (K69R, K69Q, D181R, D181N, E193V, and T197V) in conserved residues that are known or thought to be important for the activity of polo kinases. All of the mutants in the catalytic domain resulted in mitotic arrest and, in some cases, lack of septum upon overexpression (Fig. 2E,L). Immunostaining indicated that the mitotic arrest was associated with monopolar spindles in all kinase domain mutants (Fig. 2G-I). As one of the mutants (Plo1K69R) has been shown to have no or little kinase activity (Tanaka et al., 2001), this is likely to be due to dominant negative effects caused by overexpression of inactive (or less active) kinase. To test whether the mitotic arrest was due to activation of the spindle checkpoint, Plo1K69R was overproduced in the checkpoint-defective *mad2* deletion mutant. Inactivation of the spindle checkpoint completely abolished the mitotic arrest upon overexpression of kinase domain mutants, and instead gave rise to cells with large nuclei probably resulting from a continuation of the cell cycle without nuclear division (Fig. 2L).

We also examined whether this dominant negative effect is dependent on intact polo boxes. Mutations in either polo box 1 (YQL508AAA) or 3 (DHK625AAA) completely abolish the effects of overexpression of an inactive kinase Plo1K69R (Fig. 2F), confirming the importance of polo boxes for the *plo1* function.

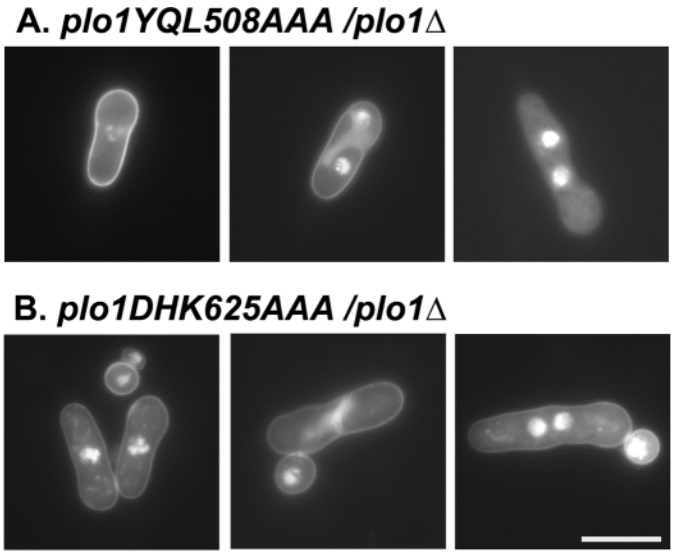

Fig. 3. Mutations in the polo boxes disrupt Plo1 function in vivo. Diploid strains were constructed in which one copy of *plo1*$^+$ is disrupted by the *his3*$^+$ gene and *plo1* mutant gene marked with the *ura4*$^+$ gene is integrated at one of the *leu1* loci. The diploids were sporulated and germinated under the selective condition (–His –Ura), which allows the growth of only the *plo1* disruptant expressing mutant *plo1* genes. Cells were fixed and stained with DAPI. (A) *plo1* disruptant cells expressing *plo1YQL508AAA*. (B) *plo1* disruptant cells expressing *plo1DHK625AAA*. Both types of cells exhibit similar cytological defects to the *plo1* disruptant. These defects include mitotic arrest with overcondensed chromosomes (left panels), disorganised septum (middle) and a failure of septation (right). Round cells seen in B are ungerminated spores. Bar, 10 μm.

Polo boxes are required for cellular function

To determine whether the *plo1* mutants we created retain function in vivo, we first tested complementation of a *plo1* disruptant using plasmid shuffling. In these experiments, we first constructed a *plo1* disruptant (*plo1::his3*$^+$) which contains a wild-type copy of *plo1*$^+$ (pURA*plo1*$^+$) on a *ura4*$^+$ marked plasmid and mutant *plo1* (pREP1*plo1**) on a *LEU2* marked plasmid. We then assayed the ability of pREP1*plo1** to support the growth of the *plo1* disruptant when pURA*plo1*$^+$ is lost (selectable by 5-FOA which kills Ura$^+$ cells).

The wild-type *plo1* gene was able to fully support the growth of a *plo1* disruptant. Various mutants in the kinase domain which we tested (Plo1K69R, K69Q, D181R, T197V, and E193V) supported little or no growth of the disruptant. Mutants in the non-catalytic domain also failed to support the growth of the disruptant (W497F, G505A, YQL508AAA, FN519AA, YM572AA, DHK625AAA, 1-633 and 1-583).

In the above experiments it was not possible to control expression levels accurately or to observe cytological phenotypes. Therefore we examined whether expression of these mutants at wild-type levels from an integrated copy can support growth of a *plo1* disruptant. The viability of *plo1* disruptants carrying integrated HA-tagged mutant *plo1* under the control of *nmt41*, which results in expression at a level comparable to the native promotor (Mulvihill et al., 1999) (Fig. 4E), were assayed through spore germination (see Materials and Methods). Wild-type *plo1* expressed in this way was able to fully complement the lethality of the disruptant. On the other hand, complementation was abolished by mutations in the kinase domain (K69R, D181R, D181N, T197V and E193V) or the non-catalytic domain (YQL508AAA, DHK625AAA, 1-633, 1-583 and 1-533). Cytological examination of germinating spores indicated that 50%-90% of disruptant cells expressing these mutant genes exhibited similar defects to those seen in a *plo1* disruptant (Fig. 3A,B). These include septation defects (either a lack of septation or the formation of defective septa) and mitotic arrest. Multiple septation or untimely septation, which is typical upon overexpression of *plo1*$^+$, was not observed. As a control, less than 5% of disruptant cells expressing the wild-type gene show abnormalities upon spore germination. It is clear then that all three polo boxes of Plo1, in addition to the catalytic domain, are required for cellular function of the protein. The fact that mutations in different polo boxes resulted in the same consequences is consistent with the idea that polo boxes together form one functional unit.

The non-catalytic domain is sufficient for cell-cycle-regulated localisation of Plo1 to the SPBs

The effect of the mutations on the cellular localisation of Plo1 protein was tested in vivo by GFP tagging of the mutant proteins at the N-terminus. Mutated *plo1* genes fused in frame with the GFP gene were expressed under the control of an attenuated *nmt1* promoter from a copy integrated at the *leu1*

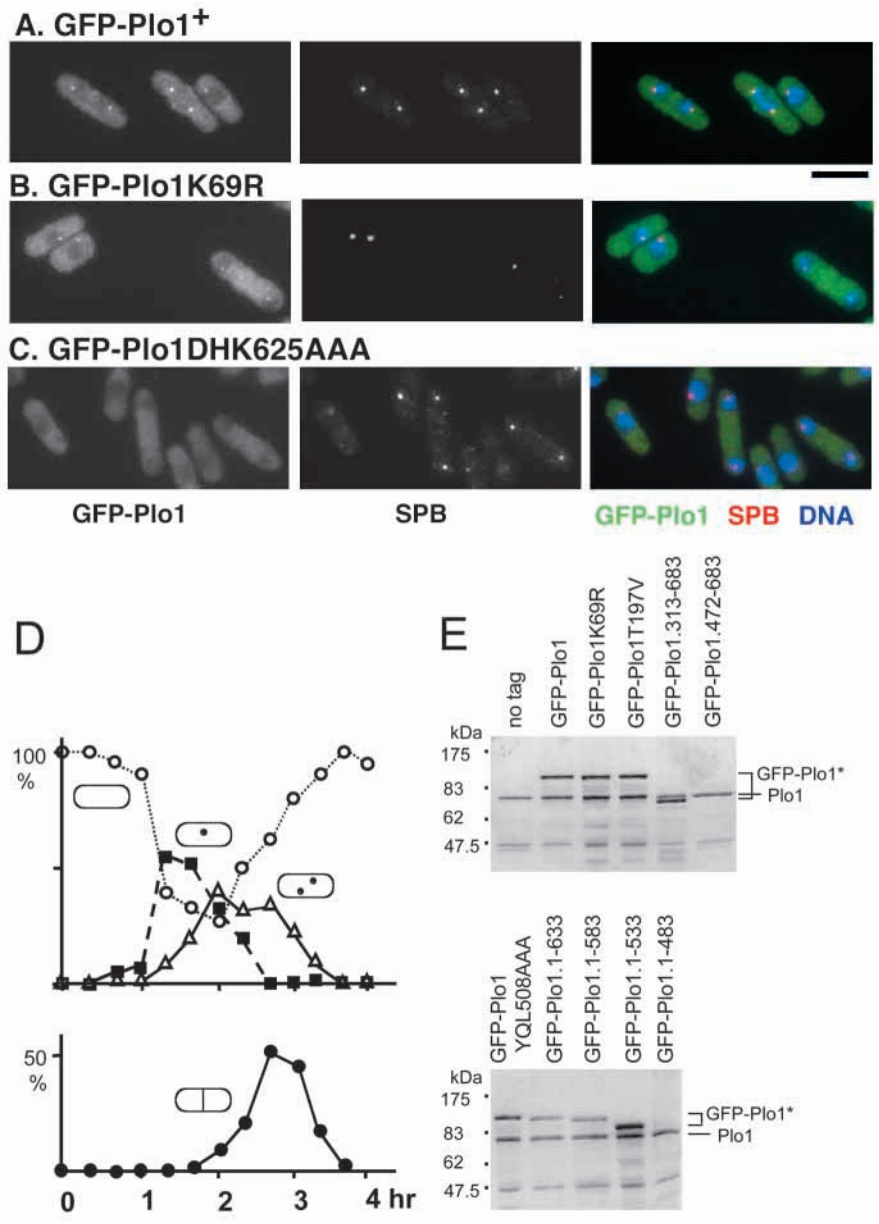

Fig. 4. The non-catalytic region is sufficient for SPB localisation. (A-C) Autofluorescence of GFP-tagged Plo1 mutant proteins (green) and immunostaining of the SPB component Sad1 (red) and DNA (blue) are shown. Co-localisation of GFP-Plo1 and Sad1 signals is seen in yellow in the merge panel. (A) GFP-Plo1$^+$ localises to the SPB in late G2 or early in mitosis before SPB separation (cell on right) and remains there throughout metaphase to anaphase (cell in middle), gradually becoming weaker late in anaphase (cell on left). Newly divided cells do not have GFP signals on SPB. Localisation to the actin ring or mitotic spindle is not detected using this construct. Bar, 10 μm. (B) GFP-Plo1K69R is indistinguishable from GFP-Plo1. (C) GFP-Plo1DHK625AAA uniformly localises to the cytoplasm throughout the cell cycle. This localisation pattern was common to all polo box point mutants and truncations. The integrity of all polo boxes is therefore required for localisation to SPBs. (D) The non-catalytic domain of Plo1 is sufficient for cell-cycle-regulated localisation to the SPBs. Small early G2 cells expressing GFP-Plo1.313-683 were collected by centrifugal elutriation and then cultured in minimal media at 30°C. Samples were taken every 20 minutes for examination of GFP signals on SPBs (one or two) under a fluorescent microscope. In the upper panel, percentages of cells with no GFP foci, one foci and two foci were represented by open circles, closed squares and open triangles, respectively. The septation index is shown in the lower panel. (E) Expression of GFP-tagged mutant Plo1 proteins assayed by western blotting using a Plo1 antibody. The amounts of the GFP-tagged proteins are comparable with endogenous Plo1 protein except Plo1.1-483 and Plo1.473-683, which were not detectable.

locus of wild-type cells. Wild-type Plo1 tagged in this way is expressed at a level comparable to the endogenous protein and displays cell cycle localisation identical to the endogenous protein (Mulvihill et al., 1999). In the case of catalytically inactive Plo1 (Plo1K69R), localisation has been shown to be unaffected by the mutation (Tanaka et al., 2001).

There were no differences observed between localisation of kinase domain mutants (GFP-Plo1K69R and T197V) and wild-type protein. Like wild-type (Fig. 4A), GFP-Plo1K69R and GFP-Plo1T197V localised initially to unseparated SPBs and remained there before becoming weaker during anaphase (Fig. 4B). This prompted us to examine whether the entire catalytic domain is dispensable for cell-cycle regulated localisation**.** We constructed a strain expressing GFP fused to the non-catalytic domain of Plo1 protein (GFP-Plo1.313-683). GFP-Plo1.313-683 localises to the SPBs in a similar manner to GFP-Plo1. To follow this localisation pattern as cells progress through the cell cycle, newly-divided short cells (early G2 cells) were collected by centrifugal elutriation. These synchronised cells were cultured and samples taken at regular time intervals. Like wild-type Plo1, GFP-Plo1.313-683 accumulates at SPBs in late G2 or very early mitosis before separation of SPBs (where only one SPB signal is visible) and remains on SPBs as they separate (two SPB signals) until late anaphase (Fig. 4D). Unfortunately, as a protein consisting of just the polo box domain of Plo1 (GFP-Plo1.472-683) was not detected by immunoblotting (Fig. 4E), it could not be determined if this region of the protein alone is sufficient for cell-cycle localisation to the SPBs. However, these results indicate that the non-catalytic domain of Plo1 is sufficient for cell-cycle-regulated localisation to the SPBs.

In contrast, localisation to the SPBs was abolished in all of the non-catalytic domain mutants tested (W497F, YQL508AAA, DHK625AAA, 1-633, 1-583, 1-533). This was not due to differences in the amount of GFP-Plo1 proteins, as all except Plo1.1-483 and Plo1.472-683 (not included in our

Table 2. Two-hybrid interactors of Plo1

Name	First residue*	Conserved motif†	Plo1	Interaction‡	
				Plo1K69R, 313-683	YQL508AAA, DHK625AAA, 1-633,1-583,1-533
Cut23	D16	TPR (174-525)	+	+	–
Dmf1/Mid1	R124		+	+	–
Sum2	M1		+	+	–
Sck1	C464	Protein kinase (302-563)	+	+	–
Abp2	A259		+	+	–
SPAC1006.03c	I270	Coiled-coil (1-40, 350-390, 480-520)	+	+	–
SPAC6B12.08	N41	DnaJ domain (5-70)	+	+	–
SPAC26H5.05	S533	Ankyrin repeat (853-938)	+	+	–

*The first residue encoded by the shortest two-hybrid clones.
†Regions containing the conserved motifs are indicated in parentheses.
‡Interaction with wild-type and Plo1 mutant proteins. (+) interaction. (–) no interaction.

cytological analysis) were detected at comparable levels to that of untagged Plo1 by immunoblotting using an antibody against the non-catalytic domain of Plo1 (Fig. 4E).

In summary, SPB localisation is dependent on the polo boxes and the non-catalytic domain of Plo1 is sufficient for its cell-cycle-regulated localisation.

Plo1 interacts with multiple proteins in a polo-box-dependent manner

Protein protein interactions may play an important role in Plo1 function. To identify proteins potentially interacting with Plo1, we carried out a two-hybrid screen of a mitoitc *S. pombe* cDNA library using full length Plo1 as a bait. Positive two-hybrid interactors included known interactors (*cut23* and *dmf1/mid1*), genes previously described in another context [*sum2*, *sck1* and *abp2* (Forbes et al., 1998; Jin et al., 1995; Sanchez et al., 1998)] and previously uncharacterised genes (Table 2). *cut23* and *dmf1/mid1* encode a subunit of the anaphase promoting complex, and a protein which localises to the pre-division site and is required for correct septum positioning, respectively. Both gene products have been shown to functionally and physically interact with Plo1 (Bahler et al., 1998; May et al., 2002). *sum2*, *sck1* and *abp2* encode a protein which may have a role in G2/M transition, a non-essential protein kinase and a putative ARS-binding protein, respectively (Forbes et al., 1998; Jin et al., 1995; Sanchez et al., 1998). The following three genes are previously uncharacterised but encode proteins which have structural motifs or limited homologies to other proteins – *SPAC1006.03c* (containing predicted coiled-coil regions), *SPAC6B12.08* (with a Dna-J domain) and *SPAC26H5.05* (containing ankyrin repeats).

Cut23 and Dmf1/Mid1 have been shown to interact with Plo1 through the non-catalytic domain (Bahler et al., 1998; May et al., 2002). To identify the region of Plo1 that mediates the interaction with each interactor we have isolated, we tested each of them against various Plo1 mutants in a directed two-hybrid assay. Without exception, these interactors were all able to interact with the dead kinase mutant (Plo1K69R) and Plo1 lacking the entire kinase domain (Plo1.313-683). On the other hand, any of the mutations in the non-catalytic domain that we tested (YQL508AAA, DHK625AAA, 1-633, 1-583, 1-533) abolished the interaction with all of the two-hybrid interactors (Table 2). This indicates that the non-catalytic domain is sufficient for the two-hybrid interactions, and that the integrity of the polo boxes is essential for interaction with all of the proteins identified in our screen. This suggests that the polo boxes together form a domain which interacts with multiple proteins.

The polo boxes are crucial for determining the specificity of protein interactions

Our site-directed mutagenesis of conserved amino acids indicated that the polo boxes are essential for the interaction with all of the proteins we examined. It is not clear, however, whether the polo boxes determine which proteins interact with Plo1. To gain an insight into this issue, we attempted to isolate mutations in *plo1* which specifically disrupt interactions with only a subset of proteins by random mutagenesis.

The entire *plo1* gene was randomly mutagenised by PCR and cloned into a bait plasmid in yeast by gap repair. In this experiment, the mutation rate assayed by sequencing was roughly 1 point mutation in every 1 kb. These mutant genes were simultaneously tested for interaction against four of the two-hybrid interactors (*cut23*, *dmf1/mid1*, *SPAC1006.03c*, and *SPAC6B12.08*) by mating individual yeast strains containing mutant *plo1* with other yeast strains containing different prey plasmids (see details in Materials and Methods).

Of 1035 potential mutants tested, 60% were positive for interaction with all (except the empty activation domain vector control), 29% did not interact with any of the prey constructs and 11% displayed differential interactions (i.e., interact with some but not others). Among mutants which displayed differential interactions, the protein interaction profiles did not show a tendency for any two of the interactors behave similarly.

To determine which residues are responsible for the specificity of the protein interactions, we sequenced some of the mutants which display differential interactions. All of these mutations mapped within or close to the polo boxes, except those disrupting the interaction with SPAC6B12.08 (Fig. 5B), indicating that the polo boxes play a crucial role in determining protein interactions. At least three mutations which disrupt interaction with Cut23 mapped in three different polo boxes, confirming the view that the polo boxes together form one domain. Those mutations which disrupt the interaction with SPAC6B12.08 mapped in a cluster of residues in subdomain X of the catalytic domain (K251E, I252T and S256P). As the entire catalytic domain including subdomain X is dispensable for the interaction with SPAC6B12.08, this may be due to stereo-hindrance caused by a structural change.

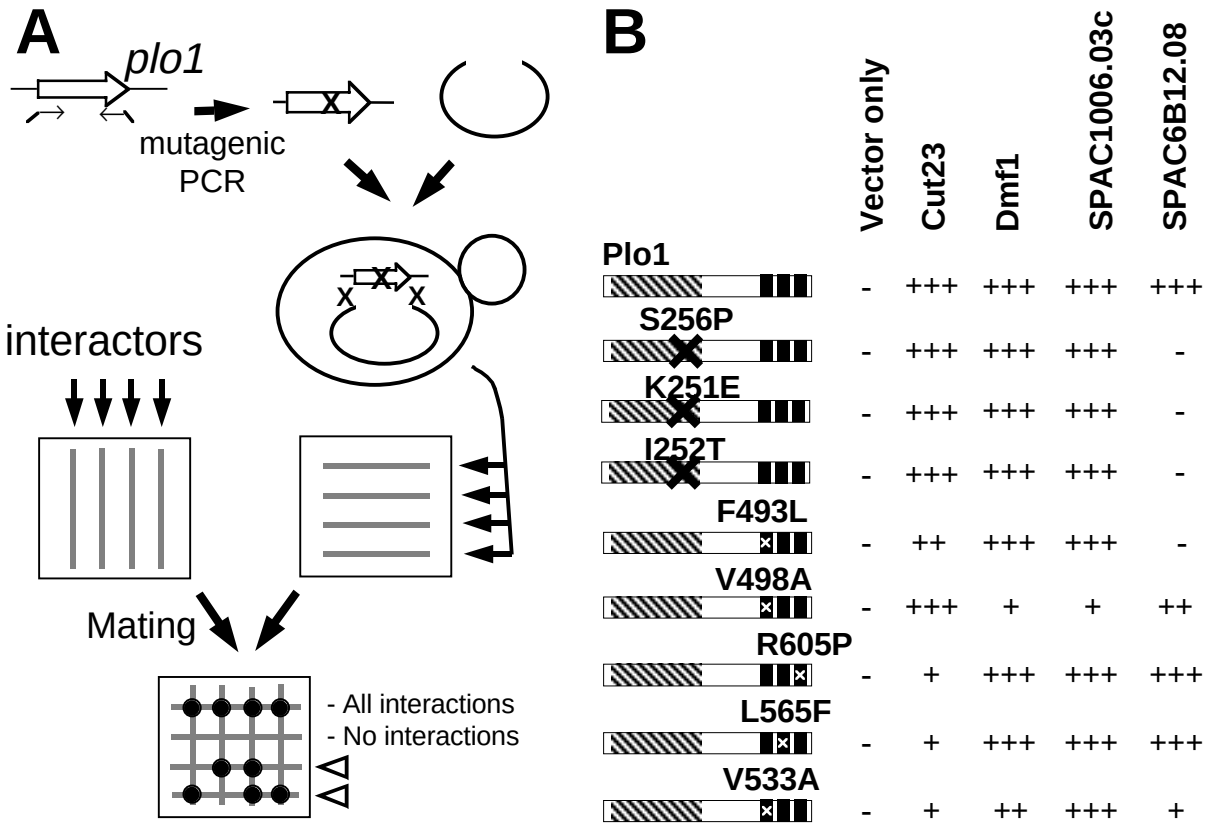

Fig. 5. Polo boxes play a crucial role in determining the protein interactions. (A) A schematic diagram of the experimental design to identify *plo1* mutants that disrupt a subset of interactions (see Materials and Methods for detail). The *plo1*$^{+}$ gene is randomly mutagenised by an error-prone PCR (about one mutation per kb). Yeast strain L40 was co-transformed with the PCR product and a gapped bait vector. Short sequences shared between the ends of the bait vector and the PCR products allow gap repair in vivo to recreate bait plasmids with various *plo1* mutations. Each strain carrying mutagenised *plo1* bait constructs was mated with Y187 strains carrying prey plasmids. The two-hybrid interaction was assessed by expression of the *HIS3* reporter gene. Plasmids were isolated from strains of interest, re-transformed to confirm the interaction pattern by expression of another reporter *lacZ*, and sequenced to determine the mutation site. (B) Sequence analysis of some Plo1 mutants that show differential interactions. Most of the mutations mapped in the polo boxes, which suggests that polo boxes are crucial for determining protein interaction. – (no or marginal interaction) < + (weak) < ++ (intermediate) < +++ (interaction at same levels as wild-type Plo1).

We hoped that mutations which differentially affect protein interactions might disrupt a subset of *plo1* functions in vivo. We tested complementation of a *plo1* disruptant by expression of these mutant genes from an integrated copy in the genome. None of these mutants were able to fully support the growth of a *plo1* disruptant. Further cytological analysis did not reveal defects specific to each of the mutants, perhaps reflecting the fact that Plo1 is likely to interact with a number of other proteins in vivo and that in no case merely a single interaction was compromised by the mutations. Nevertheless, our screen for differential two-hybrid interactions highlights the importance of the polo boxes for determining protein interactions.

Discussion

Polo boxes form one functional domain

The role of the polo boxes has been studied previously by site-directed mutagenesis in budding yeast and mammalian cultured cells (Lee et al., 1998; Lee et al., 1999; Seong et al., 2002; Song et al., 2000). In these studies, only one or a few mutations were made and a single function was assayed in vivo. Therefore it was unclear whether the non-catalytic domain containing the polo boxes consisted of functionally separable domains. In our mutational study we address this question by making a series of point mutations and truncations in the non-catalytic region, in particular at conserved residues within the polo boxes. The functions or activity of these mutants were examined by four assays – overexpression effects (two functions could be determined), complementation of a disruptant (three functions), localisation and protein interaction (with multiple interactors). All of these assays show that various mutations in the polo boxes and serial truncations from the carboxy terminus produce indistinguishable results, indicating that the polo boxes form one single functional domain.

Our functional study is consistent with a very recent structural study of the Sak polo-box domain (Leung et al., 2002). It suggests that this part of the polo-box domain (equivalent to polo box 1 and 2) autonomously folds and can interact with the second polo box domain (equivalent to polo box 3 and the C-terminal tail) to form a putative ligand-binding domain.

Polo boxes are essential for cellular function

Upon overexpression of *plo1*, mutations in the polo boxes do

not affect induction of septation but abolish the ability to interfere with spindle formation. This is the first example that polo box mutations exhibit distinct effects on two functions of polo-like kinase. The opposite effects of mutations in either the kinase domain or in the polo boxes upon overexpression of *plo1* indicate that the polo boxes are not simply required for kinase activity. In mammalian cells, carboxy terminal truncations of polo-like kinase resulted in an increase in overall kinase activity (Jang et al., 2002; Mundt et al., 1997). Our preliminary results confirmed that polo box mutants retain cell cycle regulated kinase activity in fission yeast (N.R. and H.O., unpublished).

In contrast to the overexpression assay, replacement of wild-type *plo1* gene by mutants indicated that the polo boxes are essential for at least three detectable functions of Plo1 kinase in vivo. These functional studies suggest that the catalytic and non-catalytic domains work in concert but that the requirement for the polo boxes is not simply a requirement for catalytic activity.

A role for polo boxes in localisation to centrosomes/SPBs

So what is the role of the polo boxes? It has been shown in *S. cerevisiae*, *S. pombe*, *X. laevis*, *D. melanogaster* and mammalian cultured cells that polo kinases localise to the centrosomes/SPBs in a cell cycle regulated manner and that mutations in the polo boxes abolish this localisation (Bahler et al., 1998; Golsteyn et al., 1995; Lee et al., 1998; Logarinho and Sunkel, 1998; Moutinho-Santos et al., 1999; Mulvihill et al., 1999; Shirayama et al., 1998; Song et al., 2000). In mammalian cultured cells, the non-catalytic domain alone has been shown to be sufficient for the localisation (Jang et al., 2002).

Consistently, in fission yeast it has been shown that a kinase inactive mutant can localise to the SPBs (Tanaka et al., 2001). Here we show that the polo boxes are essential, and the non-catalytic domain is sufficient for cell-cycle regulated localisation of Plo1 to the SPBs. Therefore at least one molecular role of the polo boxes is to form an autonomous domain which directs cell cycle regulation of SPB localisation.

Role of polo boxes in protein interactions

Although this and previous studies have identified a role for the polo box domain in localisation to the SPB/centrosome, our study suggests that the polo box domain is likely to play a more general role, which is to mediate interaction with multiple proteins. It has been suggested that localisation is required for polo kinase function based on the observation that mutations in the polo boxes disrupt both localisation and in vivo function (Lee et al., 1998; Song et al., 2000). In the light of our findings, more caution is necessary to interpret these results as polo box mutations simultaneously disrupt interaction with many proteins.

Site-directed mutagenesis in any individual polo box disrupts all of the functions and protein interactions of Plo1 kinase that we have examined. Therefore it is unlikely that each polo box forms a distinct subdomain which interacts with a different set of proteins. Most likely, the polo boxes together form one protein interaction domain.

Then how do the polo boxes participate in protein-protein interactions? It is possible that the polo boxes directly recognise interacting proteins and thereby determine specificity. Alternatively they may simply facilitate folding of the domain to allow intervening sequences to recognise target proteins. We have isolated mutations which disrupt only a subset of protein interactions. These mutations mapped mostly within or close to the polo boxes, despite the fact that the polo boxes occupy less than 20% of the non-catalytic domain. Therefore it is possible that the polo boxes play a crucial role in determining the specificity of protein interaction.

Plo1-interacting proteins

The two-hybrid interactors that we isolated include two previously identified interactors, Dmf1/Mid1 and Cut23. Dmf1/Mid1 is a medial ring protein required for positioning of the division site and Plo1 has been shown to interact with it and to be required for its localisation (Bahler et al., 1998). Fission yeast Cut23 is a subunit of the APC/C which interacts with Plo1, and a mutation in Plo1 which compromises that interaction fails to activate APC mediated proteolysis (May et al., 2002). Therefore at least some of the two-hybrid interactors we isolated have strong functional connections with Plo1 kinase.

Although the other two-hybrid interactors we have isolated have not yet been shown to have a clear functional relationship with Plo1 kinase, our preliminary results indicate that at least one of them (SPAC1006.3c) are indeed co-immunoprecipitated with Plo1 (N.R. and H.O., unpublished). Moreover, some studies, although limited, may suggest possible connections between some of the interactors and Plo1 function. For example, *sum2*$^{+}$ (suppressor of uncontrolled mitosis) is implicated in the G2/M transition, as it was originally isolated as a suppressor of *cdc25*$^{+}$ overproduction (Forbes et al., 1998). Abp2 was originally identified as a putative ars binding protein, but the deletion mutant shows aberrant chromatin and septal structures and fails to arrest cell cycle when replication is inhibited (Sanchez et al., 1998). Further detailed study of these two-hybrid interactors will reveal the significance of these interactions.

In other organisms, several mitotic proteins, such as human TCTP, *Drosophila* Asp, *Xenopus* Cdc25C, budding yeast septins and tubulins, have been shown to physically interact with polo kinase. TCTP is a microtubule associated protein which is phosphorylated by Plk1 kinase. Overexpression of a non-phosphorylatable form disrupts nuclear division (Yarm, 2002). Asp is another microtubule associated protein which is implicated in microtubule assembly from centrosomes (Gonzalez et al., 1990; Wakefield et al., 2001). Asp interacts physically with polo kinase and phosphorylation by polo kinase is required for its activity (do Carmo Avides et al., 2001; Gonzalez et al., 1998). Septins are required for cytokinesis in budding yeast and have been shown to interact with the budding yeast polo kinase Cdc5p both physically and functionally (Song and Lee, 2001). In most cases, these interactions were mediated entirely through the non-catalytic domain with the exceptions of the tubulins (Feng et al., 1999) and GRASP65 (Lin et al., 2000). Therefore protein interactions through the non-catalytic domain are likely to play a crucial role for polo kinase function in general.

Interactions with multiple cell cycle regulators

Then what are the roles of these protein-protein interactions?

It is possible that some of the interactors are substrates of polo kinase. As the region of the protein required for protein-protein interactions is separate from the catalytic domain, this is unlikely to be a simple substrate/kinase interaction. Rather, it is likely that physical interactions via the polo box domain act as a 'docking' mechanism to enhance the efficiency of substrate recognition. If the role of an interaction is in docking, the interactor does not have to be a direct substrate of polo kinase. The interactors can act as 'adaptors' which bring substrate and kinase together by interacting with both polo kinase and particular substrates.

It is also possible that these interactors may act as regulators to influence kinase activity directly, either positively or negatively. Indeed polo kinase is catalytically activated in a cell cycle regulated manner. However, our preliminary results suggest that Plo1 kinase which has mutated polo boxes still exhibits cell cycle regulation, suggesting its catalytic activity is regulated in other ways.

Polo kinases exhibit multiple functions at different stages of mitosis. Cell cycle regulation of kinase activity alone may not be sufficient to achieve this complex task. Interaction with multiple mitotic regulators may provide means for complex temporal and spatial regulation of polo kinase, perhaps via independent control of interaction with individual proteins. Therefore the characterisation of these interactors and an analysis of their mode of interaction will be crucial to understanding the function and regulation of polo kinase in vivo.

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