The EMBO Journal vol.15 no.6 pp.1301–1312, 1996

Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of cdc25

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The level of the mitotic activating tyrosine phosphatase cdc25 is regulated by both transcriptional and post-transcriptional mechanisms in the fission yeast *Schizosaccharomyces pombe*. We have found that cdc25 is ubiquitinated and have cloned *pub1*, a gene which regulates this event. Pub1 contains a region highly homologous to the putative catalytic domain of the human protein ubiquitin ligase E6-AP. Disruption of *pub1* elevates the level of cdc25 protein *in vivo* rendering cells relatively resistant to the cdc25-opposing tyrosine kinases wee1 and mik1. In addition, loss of wee1 activity in a *pub1*-disruption background results in a lethal premature entry into mitosis which can be rescued by loss of cdc25 function. A ubiquitin–thioester adduct of pub1 was isolated from fission yeast and disruption of *pub1* dramatically reduced ubiquitination of cdc25 *in vivo*. These results suggest that pub1 directly ubiquitinates cdc25 *in vivo*.

Keywords: cdc25/cell cycle/protein degradation/*S.pombe*/ubiquitination

Introduction

The eukaryotic cell cycle is regulated by the sequential activation of a series of cyclin-dependent kinases (CDKs) (reviewed in Norbury and Nurse, 1992; King *et al.*, 1994; Morgan, 1995). The fission yeast *Schizosaccharomyces pombe* contains a single archetypal CDK known as cdc2 (reviewed in Forsburg and Nurse, 1991). cdc2 is activated both by association with various cyclin subunits and by phosphorylation on Thr167 (Gould *et al.*, 1991; Solomon *et al.*, 1992; Fesquet *et al.*, 1993; for review see Forsburg and Nurse, 1991). The mitotic activity of cdc2 is inhibited by phosphorylation of Tyr15 by the tyrosine kinases wee1 and mik1 (Russell and Nurse, 1987; Gould and Nurse, 1989; Morla *et al.*, 1989; Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Parker *et al.*, 1992). The tyrosine phosphatase cdc25 dephosphorylates this residue, activating cdc2 and initiating mitosis (Russell and Nurse, 1986; Gould and Nurse, 1989; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Lundgren *et al.*, 1991; Millar *et al.*, 1992). Homologues of cdc25 are required for entry into mitosis in a number of different organisms, suggesting that this regulatory mechanism is well conserved (Heald *et al.*, 1993; King *et al.*, 1994).

The level of cdc25 in *S.pombe* accumulates through the cell cycle until late mitosis or G_1 when it rapidly decreases (Ducommun *et al.*, 1990; Moreno *et al.*, 1990). This pattern of accumulation is reminiscent of mitotic cyclins which are degraded by the ubiquitin system (Glotzer *et al.*, 1991; Seufert *et al.*, 1995). The ubiquitin system targets molecules for degradation by reversible covalent modification with a polyubiquitin chain (reviewed in Finley and Chau, 1991; Ciechanover, 1994). Polyubiquitinated proteins are degraded by the 26S proteasome in an ATP-dependent fashion. Protein ubiquitination involves the activation and subsequent transfer of ubiquitin to a lysine of the target molecule through a series of transiently formed thioester-ubiquitinated intermediates. Initially, an ATP-dependent high-energy thioester bond is formed between the C-terminus of ubiquitin and a cysteine residue in the ubiquitin-activating enzyme (E1). Ubiquitin is then passed in an energy-neutral reaction to a ubiquitin-conjugating enzyme (UBC) or E2, to which it is also covalently bound via a thioester linkage. Although the E2 enzymes are capable of directly ubiquitinating artificial target molecules *in vitro*, the specificity of this reaction *in vivo* appears to require a third component, a protein ubiquitin ligase (E3). To date, two protein ubiquitin ligases have been cloned, *Saccharomyces cerevisiae* UBR1 and human E6-AP (Bartel *et al.*, 1990; Huibregtse *et al.*, 1993a). These two proteins appear to function by completely different mechanisms. UBR1 seems to act as a docking site for both the relevant UBC and target molecules in N-end rule-dependent degradation (Dohmen *et al.*, 1991; Varshavsky, 1992). It has been implicated in the degradation of GPA1, the alpha subunit of a heterotrimeric G protein in *S.cerevisiae* (Madura and Varshavsky, 1994). E6-AP transfers ubiquitin from the relevant E2 to a lysine residue of its target through an E6-AP ubiquitin–thioester intermediate (Scheffner *et al.*, 1995). E6-AP binds high-risk human papillomavirus E6 oncoprotein in HPV-infected human cells (Huibregtse *et al.*, 1991). The E6–E6-AP complex binds and ubiquitinates p53. The resulting degradation of p53 is a key step in HPV-dependent oncogenesis (Crook *et al.*, 1991; Scheffner *et al.*, 1991; Wrede *et al.*, 1991; Scheffner *et al.*, 1993).

In this report we show that cdc25 is ubiquitinated in fission yeast and have cloned pub1, an E6-AP-like protein ubiquitin ligase involved in this process. Pub1 has a region highly homologous to the putative catalytic domain of E6-AP and forms a ubiquitin thioester adduct *in vivo*. Disruption of the *pub1* gene post-transcriptionally elevates cdc25 protein levels *in vivo* and dramatically reduces cdc25 ubiquitination. The data suggest that pub1 directly ubiquitinates cdc25 *in vivo*.

Results

The putative catalytic domain of E6-AP is conserved in pub1

Pub1 was fortuitously identifed in a screen originally designed to detect novel *S.pombe* tyrosine kinases (see

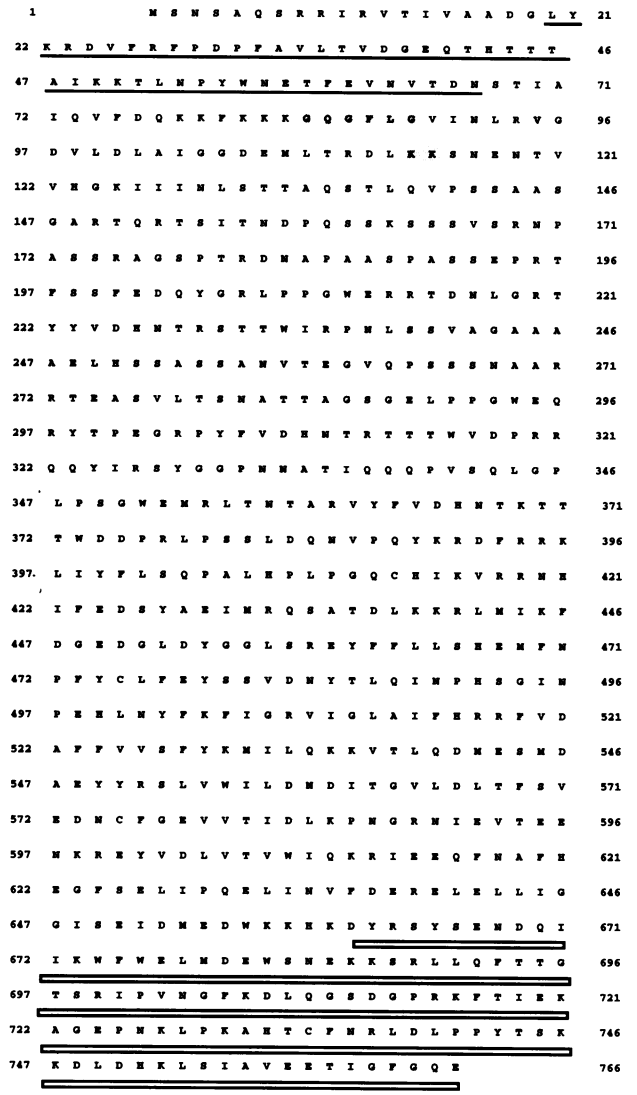

Fig. 1. The amino acid sequence of pub1. The 766 amino acid sequence deduced from the *pub1* cDNA is indicated in the conventional single letter code. The CaLB domain is underlined with a single bar. The region of homology with the putative E6-AP catalytic domain is underlined with a double bar. The putative nucleotide-binding site is shaded.

Materials and methods) (Lindberg *et al.*, 1988; Druker *et al.*, 1989; Lindberg and Pasquale, 1991). It is encoded by a single 2.9 kb message as determined by Northern blot analysis (Figure 4A). Sequence analysis of the 2847 bp *pub1* cDNA revealed a 766 amino acid open reading frame (ORF) (Figure 1). *In vitro* transcription and translation of this cDNA gave rise to an 85 kDa protein, consistent with the size of the predicted ORF (Figure 4B). The human protein ubiquitin ligase E6-AP is the most similar protein of known function in the GenBank database. It is 32% identical to pub1 (Figure 2). The protein ubiquitin ligase activity of E6-AP requires the formation of a thioester intermediate between the cysteine residue 33 amino acids from its C-terminus and the C-terminus of ubiquitin (Scheffner *et al.*, 1995). The site of E6-AP thiol ubiquitination and the region surrounding this residue are conserved in pub1 (Figures 1 and 2). Cysteine 734 is the putative site of thioester formation in pub1. Pub1 lacks the sequence required for E6 binding and has only slight similarity to the region of E6-AP shown to be required for p53 binding by deletion analysis (Huibregtse *et al.*, 1993b). A blast search of GenBank revealed three proteins of unknown function which share quite extensive homology with pub1 throughout their ORFs: the *S.cerevisiae* protein RSP5 (71% identical), the human protein D42055 (47% identical) and the mouse protein NEDD4 (40% identical) (Kumar *et al.*, 1992; Huibregtse *et al.*, 1995). The 10 proteins identified by a blast search of GenBank to be most similar to pub1 have the same conserved C-terminal domain found in both E6-AP and pub1 (Figure 1). These proteins may define an E6-AP-like family of protein ubiquitin ligases (Scheffner *et al.*, 1995).

Further examination of the pub1 sequence revealed both a Ca^{2+}-dependent lipid-binding domain (CaLB) and a putative ATP-binding site near the N-terminus (Figures 1 and 3). The CaLB domain is a sequence motif conserved in a set of proteins which translocate to phospholipid membranes in a Ca^{2+}-dependent fashion *in vivo* including protein kinase C (PKC), the Ras GTPase activating protein (GAP) and cytosolic phospholipase A_2 (Figure 3) (Clark *et al.*, 1991). We have found that a purified fusion protein between maltose-binding protein and pub1 dramatically increases its affinity for hydrophobic column matrices in the presence of Ca^{2+} (data not shown). This suggests that pub1 may also translocate to a phospholipid membrane in a Ca^{2+}-dependent fashion *in vivo*.

The *pub1* gene was physically mapped by probing a collection of contiguous cosmid clones spanning the *S.pombe* genome with *pub1* cDNA (Mizukami *et al.*, 1993). Three overlapping cosmids, 323, 437 and 1187, hybridized to our probe indicating that *pub1* is on the right arm of chromosome 1 near the centromere. Two adjacent *Not*I sites and *cut7* are the closest distal markers to the gene.

A *pub1* disruption was constructed by replacing a 948 bp *Sal*I–*Nsi*I fragment of the *pub1* ORF with the *ura4* gene (Figure 4D). This construct effectively disrupts about two thirds of the *pub1* ORF including the putative protein ubiquitin ligase domain. A linear fragment containing the disrupted *pub1* gene was introduced into the diploid strain SP 826 (Table I). Stable Ura^+ transformants were recovered by screening for 5-fluoroorotic acid (FOA) sensitivity and Southern blot analysis confirmed that most of those carried one copy of the *pub1*-disruption allele (*pub1::ura4*) and one copy of the wild-type gene (data not shown). The diploid heterozygous for the *pub1* disruption produced four viable spores indicating that pub1 is not essential for vegetative growth. Southern blot analysis of the Ura^+ haploid cells confirmed that they carried only the disrupted *pub1* gene (Figure 4C).

Genetic interactions with wee1 and cdc25

Cells bearing a *pub1* disruption divide at a cell size noticeably smaller than wild-type. A similar, though more pronounced phenotype can be observed in cells which have lost wee1 function or are overproducing cdc25 (Nurse, 1975; Russell and Nurse, 1987). To determine if pub1 regulated either cdc25 or wee1 activity we tested for an interaction between pub1 and these genes (Table II).

wee1-50 is a temperature-sensitive recessive loss-of-function allele of the non-essential gene *wee1* (Nurse, 1975; Nurse and Thuriaux, 1980). At restrictive tempera-

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pub1   416 KVRRNHIFED--SYAE-IMRQSATDLKKRLMIKFDGEDGLDYGGLSREYFFLL 465
E6-AP  525 KVRRDHIIDDALVRLEMIAMENPADLKKQLYVEFEGEQGVDEGGVSKEFFQLV 577

pub1   466 SHEMFNPFYCLFEYSSVDNYTLQINPHSGINPEHLNYFKFIGRVIGLAIFHRR 518
E6-AP  578 VEEIFNPDIGMFTY-DESTKLFWFNP-S--SFETEGQFTLIGIVLGLAIYNNC 626

pub1   519 FVDAFFVVSFYKMILQKKVTLQDMESMDAEYYRSLVWILDNDITGVLD--LTF 569
E6-AP  627 ILDVHFPMVVYRKLMGKKGLFVDLGDSHPVLYQSLKDLLEYVGNVEDDMMITF 679

pub1   570 -SVEDNCFGEVVTIDLKPNGRNIEVTEENKREYVDLVTVWI-QKRIEEQFNAF 620
E6-AP  680 QISQTNLFGNPMMYDLKENGDKIPITNENRKEFVNLYSDYILNKSVEKQFKAF 732

pub1   621 HEGF-SELIPQELINVFDERELELLIGGISEIDMEDWKKHTDY-RSYSENDQI 671
E6-AP  733 RRGFHMVTNESPLKYLFRPEEIELLICGSRNLDFQALEETTEYDGGYTRDSVL 785

pub1   672 IKWFWELMDEWSNEKKSRLLQFTTGTSRIPVNGFKDLQGSDGPRKFTIEKAG- 723
E6-AP  786 IREFWEIVHSFTDEQKRLFLQFTTGTDRAPV-G-----G-LGKLKMIIAKNGP 831
                          *
pub1   724 EPNKLPKAHTCFNRLDLPPYTSKKDLDHKLSIAVEETIGFGQE           766
E6-AP  832 DTERLPTSHTCFNVLLLPEYSSKEKLKERLLKAITYAKGFGML           875
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Fig. 2. Sequence comparison of fission yeast pub1 and human protein ubiquitin ligase E6-AP. An amino acid sequence alignment of the pub1 and E6-AP C-termini by the Needleman–Wunsch method (Intelligenetics) is shown. Identical amino acids appearing in both sequences are shaded. The putative site of pub1 thioester ubiquitination (Cys734) is indicated by an asterisk. The residues of E6-AP are numbered according to its GenBank file.

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PUB1    LYKRDVFRFPDPFAVLTV--DGE--QTHITTAIK-KTLNFYWNET-FEVNVTDN
CPLA2   M-----LDTPDPYVELFIS--TTPDSRKRTRHFN-NDIDPVWNET-FEFIL-DP
PKCg    M---DPNGLSDPYVKLKLIPDPRNLTKQKTRTVK-ATLNFVWNET-FVFNI-KP
P65a    M-----GGTSDPYVKVFLL--PEKKKKFETKVHR-KTLNPVFNEQ-FTFKV--P
P65b    M---DVGGLSDPYVKIHLMQNGKRLKKKKTTIKK-HTLNPYYNEA-FSFEV--P
GAP     L---PVKHFTNPYCNIYLN----SVQVAKTHAR--BGQNPVWAEA-FVRDD-LP
PLCg1   L-PNRGRGIVCPFVEIEVAGAEYDSIKQKTEFVVDNGLNPVWPAKPFHFQISNP

consensus  M------G-SDPYV-L-L--------K-KTR--K--TLNPVWNE--F-F-V--P
                 T  FA I V        R R K  R    I   F         I
                      V I             H         Y         L
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Fig. 3. CaLB domain comparison. The putative CaLB domain of pub1 is compared with those of six mammalian proteins and a consensus sequence is determined. Invariant residues are indicated with bold letters. Conserved regions are shaded and gaps are indicated by a dash at the appropriate residue position. The conservative amino acid changes between proteins are indicated in the consensus sequence.

ture cells bearing a *wee1-50* allele undergo mitosis at a cell size significantly smaller than wild-type. Loss of wee1 function in a *pub1*-disruption background is lethal (Figure 5A). The apparent fragmentation of chromosomes, formation of enucleate cells and occasional septation through the nucleus observed in the double mutant (SP 1209) at restrictive temperature closely resembles the mitotic catastrophe phenotype thought to be the result of premature entry into mitosis (Figure 5B) (Russell and Nurse, 1987; Lundgren *et al.*, 1991). A similar mitotic lethality with *wee1-50* has been observed both in the absence of mik1 and in cells overproducing cdc25 (Russell and Nurse, 1986, 1987; Lundgren *et al.*, 1991).

If disruption of *pub1* increases cdc25 activity *in vivo*, loss of cdc25 may rescue the *pub1::ura4 wee1-50* synthetic lethality. To test this, a *pub1::ura4 wee1-50 cdc25-22* triple mutant (SP 1211) was constructed. *cdc25-22* is a temperature-sensitive, recessive, loss-of-function allele of *cdc25*. The *pub1::ura4 wee1-50 cdc25-22* triple mutant was viable at restrictive temperature indicating that the mitotic lethality of the *pub1::ura4 wee1-50* double mutant requires cdc25 function (Figure 5). In contrast, the *mik1::ura4 wee1-50 cdc25-22* triple mutant undergoes lethal premature mitosis at restrictive temperature (Lundgren *et al.*, 1991).

To investigate the interaction between pub1 and cdc25 further, a *pub1::ura4 cdc25-22* double mutant (SP 1210) was constructed. At restrictive temperature, this double mutant arrests at the G_2/M boundary with the same terminal phenotype as a *cdc25-22* single mutant (SP 628), i.e. elongated cells with a 2N DNA content (Figure 5). The *cdc25-22* mutation can be rescued both by overexpression of tyrosine phosphatase activity and by loss of wee1 function (Fantes, 1979). If disruption of *pub1* is either increasing the activity of a tyrosine phosphatase other than cdc25 or inhibiting wee1, it is insufficient to rescue *cdc25-22*.

Pub1 disruption increases tolerance of wee1 and mik1 overproduction

If disruption of *pub1* increases cdc25 activity *in vivo*, it should increase the resistance of cells to both wee1 and mik1 activity. To test this, we overproduced wee1 and mik1 under the control of the thiamin-repressible *nmt* promoter in both wild-type and *pub1*-disrupted cells (Maudrell, 1993). The pREP1 plasmid contains a wild-type *nmt* promoter and the pREP41 plasmid a mutated version which reduces the level of expression ~10-fold. Expression of either *wee1* or *mik1* from pREP41 on thiamin-free media arrested wild-type *S.pombe* at the G_2/M boundary preventing colony formation (Table II and Figure 6). In contrast, *pub1::ura4* cells expressing wee1 or mik1 from the same vector could readily form colonies in the absence of thiamin (Table II and Figure 6). The level of both wee1 and mik1 expression was the same in both wild-type and *pub1*-disrupted strains as determined by Western blot analysis (data not shown). Although more resistant than wild-type, *pub1*-disrupted cells were still sensitive to increased levels of wee1 and mik1. Expression of either kinase from a pREP41 vector increased the size at which these cells divide (data not shown). Increasing *wee1* or *mik1* expression still further using a pREP1 vector arrested *pub1*-disrupted cells, preventing formation of colonies on thiamin-free media (Figure 6) (Maudrell, 1993).

Pub1-disruption elevates cdc25 protein levels

The genetic evidence suggested that disruption of *pub1* increases cdc25 activity *in vivo*. To determine if cdc25 protein levels were affected we examined cdc25 levels in lysates prepared from asynchronous cultures of both wild-type and *pub1*::*ura4* strains by Western blot analysis (Figure 7). cdc25 disruption and overproducing strains were used as controls for antibody specificity. The level of cdc25 in asynchronously growing *pub1::ura4* cells is

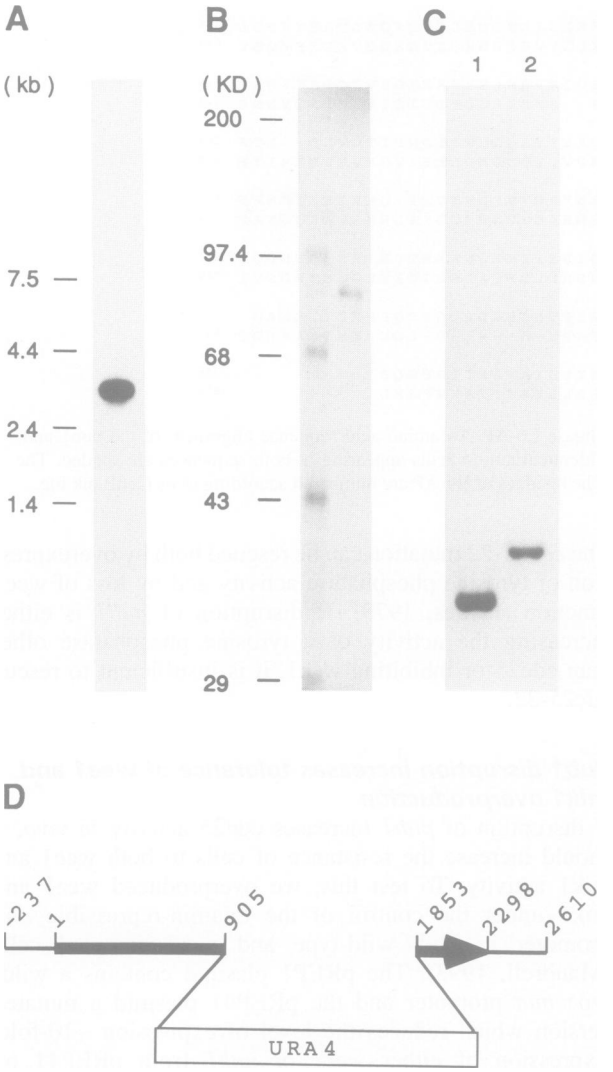

Fig. 4. The pub1 transcript, translation product and deletion. (**A**) A Northern blot of total RNA isolated from wild-type (972) *S.pombe* cells grown to mid-log in YEA was probed with *pub1* cDNA. The positions of the RNA molecular weight standards used are indicated on the left. (**B**) The *pub1* cDNA cloned into pBluescript SK– was transcribed and translated *in vitro* in the presence of [^{35}S]methionine. The translation mixture was subjected to electrophoresis in a 10% Laemmli gel alongside ^{14}C-labeled molecular weight standards and the gel was autoradiographed. The sizes of the molecular weight standards used are indicated on the left. (**C**) Genomic DNA isolated from wild-type (972) (lane 1) and *pub1*-disrupted haploid cells (SP 1207) (lane 2) was digested with *Eco*RI and probed with the 2.1 kb *pub1 Eco*RI fragment. (**D**) A schematic of the *pub1* cDNA disruption construct is shown. The *pub1* ORF is indicated by the solid black arrow, the deleted sequence by the gap and the corresponding nucleotide numbers are shown above.

~4-fold higher than in wild-type cells (Figure 7, lanes 1 and 2). Loss of pub1 did not affect the steady state level of *cdc25* message (data not shown). Thus, the disruption of *pub1* post-transcriptionally increases the level of cdc25 protein *in vivo,* indicating that pub1 could act by inhibiting cdc25 translation or enhancing its degradation.

Cdc25 is ubiquitinated in a pub1-dependent fashion

The sequence similarity between pub1 and the protein ubiquitin ligase E6-AP raised the possibility that pub1 might enhance cdc25 degradation by ubiquitination. To determine if cdc25 was ubiquitinated in *S.pombe*, a strain was constructed bearing both hemagglutinin-tagged cdc25 in place of the wild-type gene and the *mts2* mutation. *mts2* is a temperature-sensitive mutant in the S4 subunit of the 26S proteasome (Gordon *et al.*, 1993). The 26S proteasome is responsible for the degradation of protein ubiquitin conjugates, and these conjugates accumulate in a *mts2* mutant (Finley and Chau, 1991; Gordon *et al.*,

Table I. List of *S.pombe* strains

Strain	Genotype
972	h^{-S}
SP 6	h^{-S} *leu1-32*
SP 546	h^{+N} *wee1-50*
SP 628	h^{+N} *cdc25-22 leu1-32*
SP 826	h^{+N}/h^{+N} *ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ ura4-D18*
SP 974	h^{+N} *cdc2-3w* cdc*25::ura4 ura4-D18*
SP 1062	h^{-S} *cdc25-22*ΔART8*-25.4 leu1-32 ura4-*
SP 1207	h^{-S} *pub1::ura4 ura4-D18*
SP 1208	h^{+N} *pub1::ura4 leu1-32 ura4-D18*
SP 1209	h^{-S} *pub1::ura4 wee1-50 ura4-D18*
SP 1210	h^{-S} *pub1::ura4 cdc25-22 ura4-D18*
SP 1211	h^{-S} *pub1::ura4 wee1-50 cdc25-22 ura4-D18*
SP 1212	h^{-S} *mts2 cdc25*$(HA)_3$ *leu1-32*
SP 1213	h^{-S} *mts2 pub1::ura4 cdc25*$(HA)_3$ *leu1-32 ura4-D18*

Strain SP 1062 was constructed by stably transforming SP 586 h^{-S} *cdc25-22, leu1*-32, *ura4*- with pART8-25.4 which carries *cdc25* under the strong constitiutive ADH promoter control.
Strain CFX 109-2R h^{+N} *cdc2*$(HA)_3$, *leu1–32, ura4-D18* used to construct strains SP 1212 and SP 1213 was a generous gift from D.Conklin. The *cdc*25 allele *cdc25*$(HA)_3$ has a triple tandem hemagglutinin epitope inserted in-frame at the *Cla*I site of *cdc25*. The resulting construct was integrated into the *S.pombe* genome at the *cdc25-22* site. This allele rescues *cdc25-22* at restrictive temperature (D.Conklin, unpublished results).
The *mts2* allele used to construct strains SP 1212 and SP 1213 was a generous gift from N.Hastie.

Table II. Genetic interactions

(A)

Relevant genotype	25°C	30°C	37°C
pub1::ura4	+	+	+
wee1-50	+	+	+
pub1::ura4, wee1-50	+	+	–(l.m.)
cdc25-22	+	+	–(*cdc*$^-$)
pub1::ura4, cdc25-22	+	+	–(*cdc*$^-$)
pub1::ura4, wee1-50, cdc25-22	+	+	+

(B)

Relevant genotype	+Thiamin	–Thiamin
pub1$^+$ + pREP41	+	+
pub1$^+$ + pREP41*wee1*	+	–(*cdc*$^-$)
pub1$^+$ + pREP41*mik1*	+	–(*cdc*$^-$)
pub1::ura4 + pREP41	+	+
pub1::ura4 + pREP41*wee1*	+	+
pub1::ura4 + pREP41*mik1*	+	+
pub1::ura4 + pREP1	+	+
pub1::ura4 + pREP41 *wee1*	+	–(*cdc*$^-$)
pub1::ura4 + pREP1*mik*1	+	–(*cdc*$^-$)

l.m., lethal mitosis
cdc$^-$, cell cycle arrest with single nuclei and elongated cells. These cells arrest at the G_2/M boundary with a 2N DNA content.

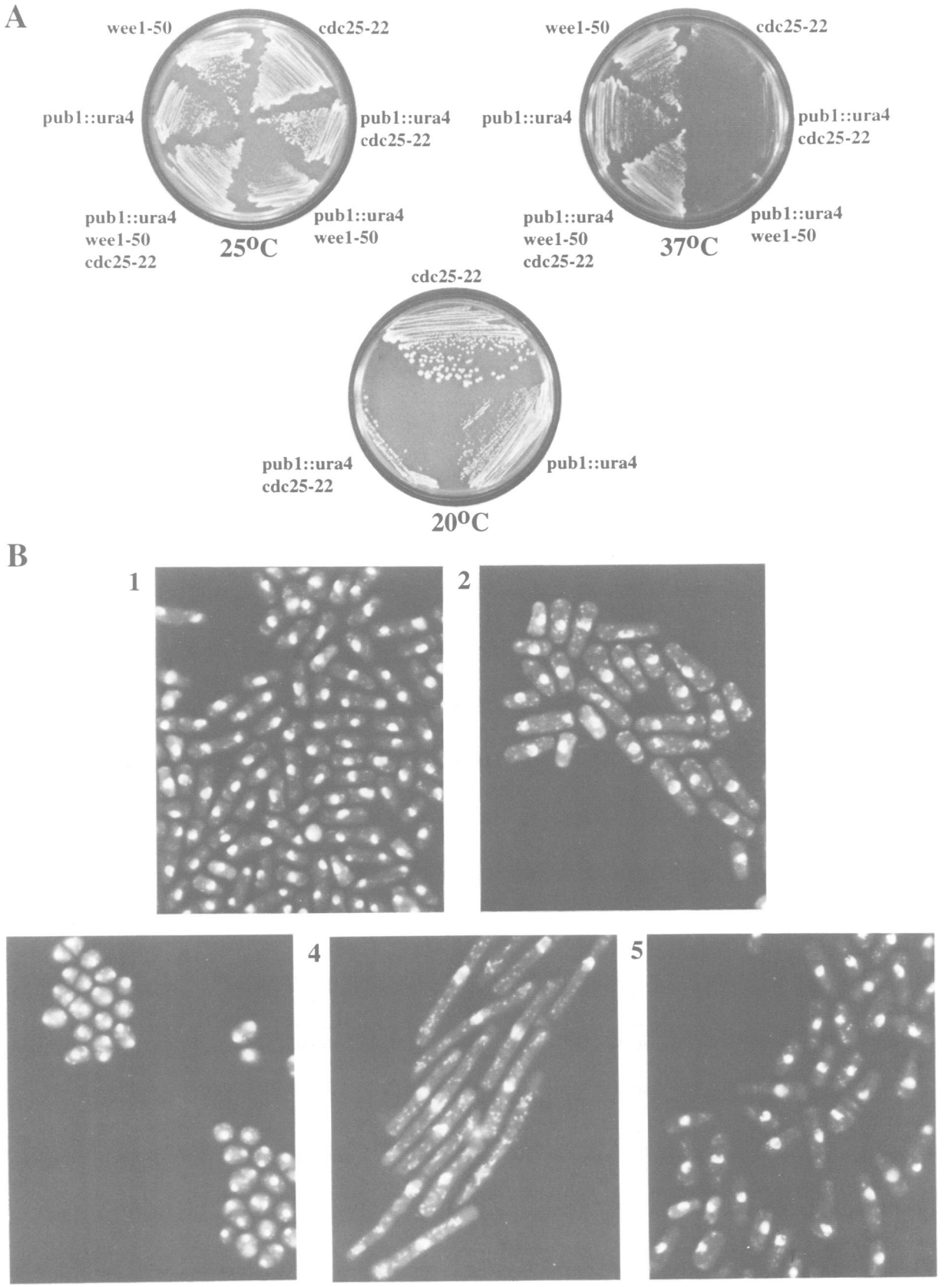

Fig. 5. Pub1 interacts genetically with wee1 and cdc25. (**A**) Three plates were streaked with strains of the indicated genotypes: *pub1::ura4* (SP1207); *wee1-50* (SP 546); *cdc25-22* (SP 628); *pub1::ura4 cdc25-22* (SP 1210); *pub1::ura4 wee1-50* (SP 1209); *pub1::ura4 cdc25-22 wee1-50* (SP1211). One of the plates was incubated at 20°C for 14 days, another at 25°C for 5 days and the last at 37°C for 5 days. (**B**) Visualization by Dapi staining of: (1) *pub1::ura4* cells (SP 1207) grown at 25°C; (2) *pub1::ura4* cells (SP1207) grown at 37°C; (3) *pub1::ura4 wee1-50* cells (SP 1209) grown to mid-log at 25°C and then incubated at 37°C for 3 h; (4) *pub1::ura4 cdc25-22* cells (SP 1210) grown to mid-log at 25°C and then incubated at 37°C for 3 h; (5) *pub1::ura4 wee1-50 cdc25-22* (SP 1211) grown to mid-log at 25°C and then incubated for 3 h at 37°C.

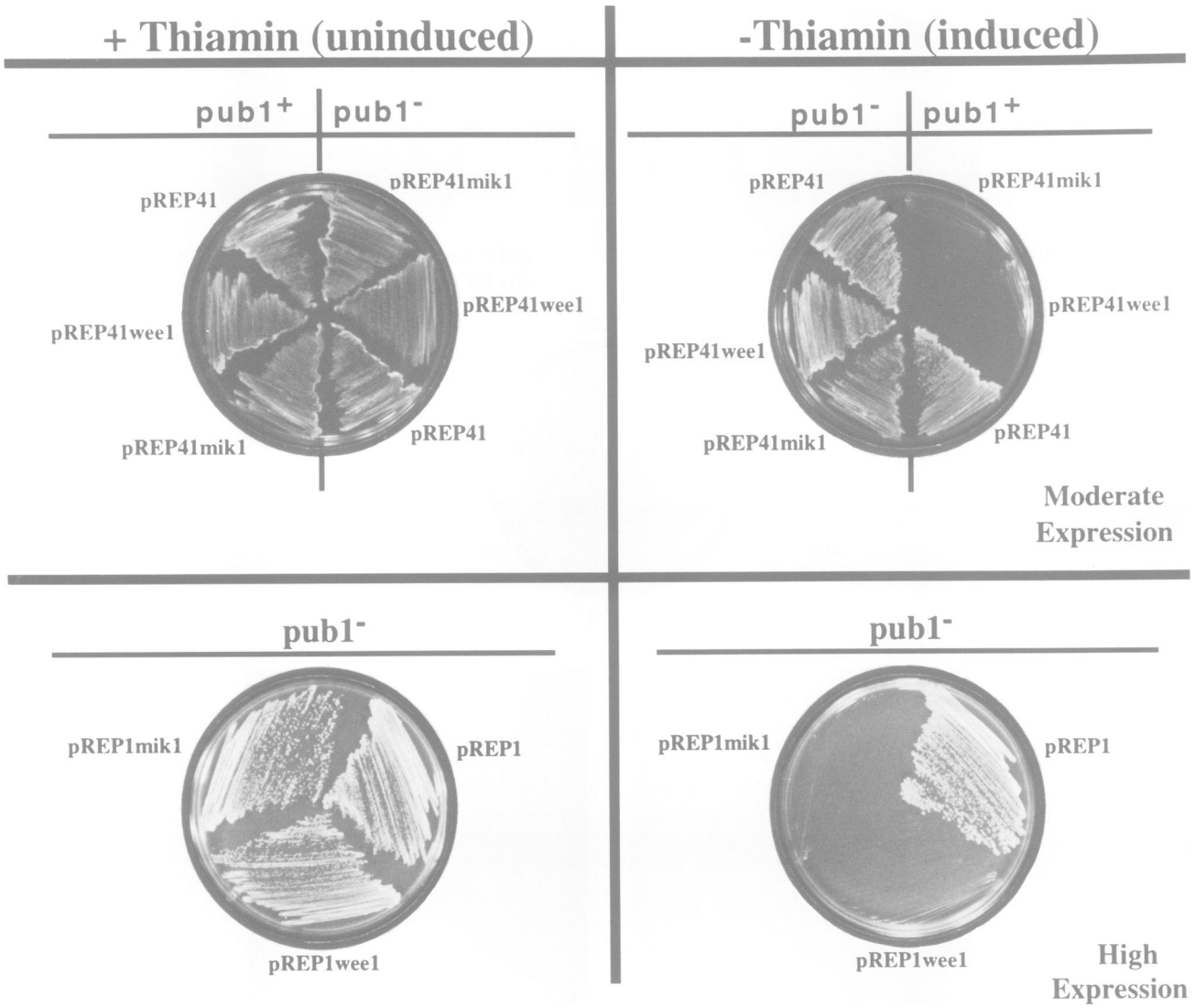

Fig. 6. Disruption of pub1 increases tolerance of wee1 and mik1 expression. A wild-type *pub1* strain (SP6) and a *pub1*-disruption strain (SP1208) containing the indicated plasmids were streaked onto either PM + 20 μM thiamin or PM as shown. The plates were then incubated at 30°C for 6 days.

1993). At both permissive and restrictive temperatures, *mts2* mutants accumulate a ladder of higher molecular weight species of cdc25 (Figure 8A, lanes 1–4). cdc25 immunoprecipitates from this strain were analyzed by Western blotting for the presence of ubiquitin. The ladder of higher molecular weight cdc25 species which accumulate in an *mts2* mutant cross-react with an anti-ubiquitin antibody, indicating that they are ubiquitinated forms of cdc25 (Figure 8B, lanes 2 and 4). The anti-ubiquitin antibody preferentially recognizes the higher molecular weight forms of ubiquitinated cdc25 consistent with multiple ubiquitination. No immunological cross-reactivity was detected in isogenic control strains lacking hemagglutinin epitope-tagged cdc25 (data not shown).

To determine if pub1 was involved in ubiquitination of cdc25, we constructed a pub1-disruption *mts2* strain with hemagglutinin-tagged cdc25 in place of the wild-type gene (SP 1213). Both the higher molecular weight forms of cdc25 and the anti-ubiquitin cross-reactive species are absent in a *pub1*-deletion background, indicating that the predominant mechanism for ubiquitination of cdc25 requires pub1 (Figure 8A, lanes 5–8, and 8B, lanes 1 and 3). With extended exposure however, a faint ladder of cdc25 higher molecular weight species can be seen in a *pub1*-disruption background. We estimate that disruption of *pub1* reduces the accumulation of cdc25 higher molecular weight species 10-fold (data not shown).

Pub1 is thiol ubiquitinated in vivo

The conservation of the putative E6-AP catalytic domain in pub1 suggested that pub1 may also employ a thiol ubiquitinated intermediate during catalysis (Scheffner *et al.*, 1993, 1995). We were unable to detect this intermediate in logarithmically growing cells, presumably because it is both unstable and most probably present at low levels. We reasoned that in the absence of target molecules, the components of the ubiquitin cycle which employ ubiquitin thioester intermediates may accumulate in their ubiquitin-charged intermediate form. The formation of new target molecules was blocked by inhibition

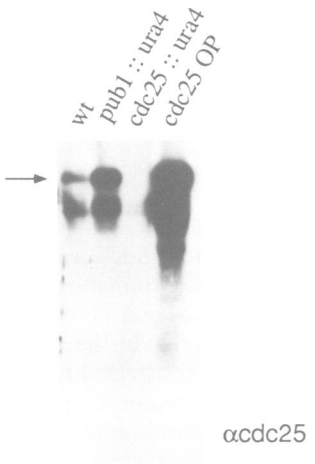

Fig. 7. Disruption of *pub1* increases the level of cdc25 protein *in vivo*. 100 μg of soluble protein from (lane 1) wild-type cells (972); (lane 2) pub1-disrupted cells (SP 1207); (lane 3) cdc25 disrupted cells (SP 974); and (lane 4) cells overproducing cdc25 (SP 1062); grown to mid-log in YEA at 30°C were resolved by SDS–PAGE on a 10% Laemmli gel. The resolved proteins were transferred to nitrocellulose and probed with an affinity purified anti-cdc25 polyclonal antibody.

of translation with cycloheximide followed by a brief incubation to permit the degradation of all accessible target molecules. Since it is recycled, blocking translation should not deplete the cellular stores of ubiquitin (Finley and Chau, 1991).

If thiol-ubiquitinated pub1 accumulates in cycloheximide-treated cells it should be possible to co-immunoprecipitate the two proteins in a cycloheximide-dependent fashion. In addition, if pub1 and ubiquitin are linked by a thioester bond, their association should be both refractile to denaturing reagents and labile to reducing reagents. Both hemagglutinin-tagged and untagged pub1 under the control of the *nmt* promoter in pREP1 were introduced into a *pub1*-disruption strain. Cultures of both strains were shifted to thiamin-free medium for 10 h to induce *pub1* expression after which cycloheximide was added. Lysates were prepared in the absence of reducing agents from both cultures immediately before and 1 h after the addition of cycloheximide. The cells were lysed under strongly denaturing conditions [1% lithium lauryl sulfate (LDS)] to disassociate non-covalent protein interactions. Pub1 was immunoprecipitated from dilutions of these lysates and the immunoprecipitates divided into two aliquots. One aliquot was incubated and washed in RIPA buffer containing 20 mM DTT to displace and remove thioester bound ubiquitin and the other was treated identically with RIPA buffer lacking DTT. The immunoprecipitates were then incubated in Laemmli sample buffer containing β-mercaptoethanol (β-me) and subjected to electrophoresis on an 8% gel to resolve pub1 and on an 18% gel to resolve any ubiquitin released by β-me treatment of the samples. The gels were then analyzed by Western blot analysis for the presence of pub1 (Figure 9C) and ubiquitin (Figure 9D). One hour after treating the cells with cycloheximide, ubiquitin co-immunoprecipitated with pub1 (Figure 9C and D, lane 4). The absence of ubiquitin in the cycloheximide-treated untagged pub1 control demonstrated that the co-immunoprecipitation of ubiquitin with pub1 is specific (Figure 9C and D, lane 5). The association

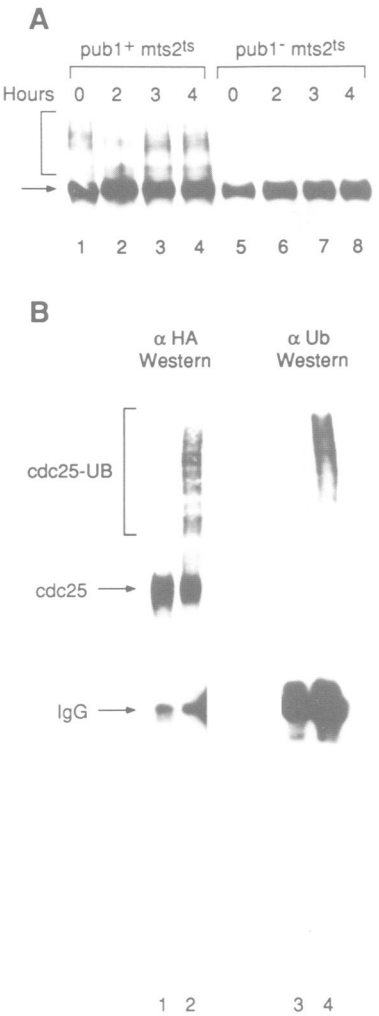

Fig. 8. Pub1 is involved in cdc25 ubiquitination. (**A**) Cultures of both *pub1*$^+$ cells (SP 1212) *h*$^{-s}$ *mts2,cdc25(HA)*$_3$ *leu1-32* and *pub1*$^-$ cells (SP 1213) *h*$^{-s}$ *pub1::ura4 mts2 cdc25(HA)*$_3$ *leu1-32 ura4D-18* were grown to mid-log phase at 25°C and then shifted to 37°C. Cell lysates were prepared from both strains after 0, 2, 3 and 4 h at 37°C. 100 μg of soluble protein from each lysate was resolved on an 8% Laemmli gel, transferred to nitrocellulose and probed with the 12CA5 anti-HA monoclonal antibody to detect cdc25. (**B**) Strains SP 1212 and SP 1213 grown to mid-log phase at 25°C were incubated at 37°C for 3 h and lysed. cdc25 was immunoprecipitated from each lysate with the affinity purified anti-cdc25 antibody and the immunoprecipitated proteins resolved on an 8% Laemmli gel. Each sample was run in duplicate: lanes 1 and 3 contain immunoprecipitates from *pub1*$^-$ SP 1213 and lanes 2 and 4 contain immunoprecipitates from *pub1*$^+$ SP 1212. Lanes 1 and 2 were probed with the 12CA5 anti-HA monoclonal antibody to detect the total cdc25 present, and lanes 3 and 4 were probed with an anti-ubiquitin polyclonal antibody to detect ubiquitinated cdc25.

of ubiquitin with pub1 was sensitive to reducing agents. Incubating and washing the cycloheximide-treated pub1 immunoprecipitate with DTT removed the bound ubiquitin (Figure 9C and D, lane 2). In the absence of DTT treatment, ubiquitin remains bound to pub1 until released by the β-me in the sample buffer (Figure 9C and D, lane 4). Like all thioesters, the association between pub1 and ubiquitin was also sensitive to 0.1 M NaOH and refractile to 1 M formic acid (data not shown) (Scheffner *et al.*, 1995).

If pub1 is thiol ubiquitinated, the association between

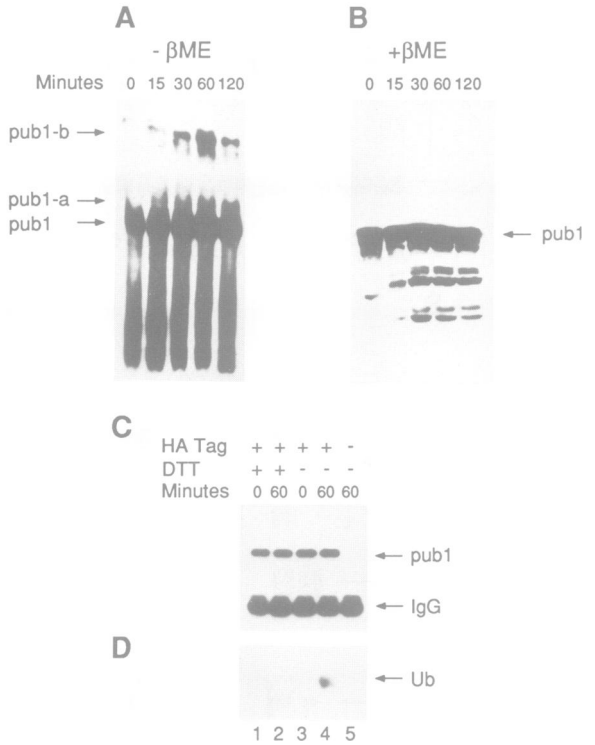

Fig. 9. Pub1 is thioester ubiquitnated *in vivo*. (**A** and **B**) C-terminally HA-tagged pub1 was induced in a SP 1208 + pREP1*pub1*/HA culture for 10 h at 30°C in PM and then cycloheximide was added. Aliquots of the culture were removed at 0, 15, 30, 60 and 120 min after addition of cycloheximide and lysates prepared in the absence of reducing agents. PAGE samples were then prepared both in the absence (A), and in the presence (B) of β-me PAGE samples containing 50 μg of protein prepared in the absence of β-me were subjected to electrophoresis on a modified 8% Laemmli gel at 4°C. SDS–PAGE samples containing 50 μg of protein prepared in the presence of β-me were subjected to electrophoresis in an 8% Laemmli gel at room temperature. The proteins in both gels were transferred to nitrocellulose and probed with 12CA5 anti-HA monoclonal antibody to detect pub1. (**C** and **D**) SP 1208 + pREP1*pub1*/HA and SP 1208 + pREP1*pub1* were grown for 10 h at 30°C in the absence of thiamin to induce *pub1* expression. Cycloheximide was then added and incubation continued for another hour. Lysates were prepared in the absence of reducing agents from both strains immediately before and 60 min after the addition of cycloheximide. The 12CA5 anti-HA monoclonal antibody was used to immunoprecipitate pub1 and the immunoprecipitates were divided into two aliquots. One aliquot was incubated for 1 h at 4°C in RIPA buffer containing 20 mM DTT and then washed in the same buffer (+ DTT). The other aliquot was treated identically with RIPA buffer lacking DTT (–DTT). The immunoprecipitates were subjected to electrophoresis both on an 8% Laemmli gel to resolve pub1 (C) and on an 18% Laemmli gel to resolve ubiquitin released by treament of thioester ubiquitinated pub1 with the β-me-containing sample buffer (D). The proteins in both gels were transferred to nitrocellulose and probed with either 12CA5 anti-HA antibody for the presence of pub1 (C) or an anti-ubiquitin polyclonal antibody for the presence of free ubiquitin (D).

pub1 and ubiquitin should be coincident with the appearance of a DTT-sensitive, 5–10 kDa higher molecular weight form of pub1. To test this, *pub1*-deletion strains carrying either hemagglutinin-tagged or untagged pub1 in pREP1 were shifted to thiamin-free medium for 10 h to induce *pub1* expression. Cycloheximide was then added and aliquots removed from each culture at the indicated times. Gel samples of lysates from each time point were prepared in both the presence and absence of β-me and subjected to Western blot analysis using the anti-HA monoclonal antibody 12CA5 to detect pub1. Two more slowly migrating forms of pub1 (a and b) accumulated in cycloheximide-treated cells bearing pREP1*pub1*/HA (Figure 9A). In the presence of reducing agents, both species are absent (Figure 9B). The decrease in mobility exhibited by pub1-a could be accounted for by the addition of a single molecule of ubiquitin. Pub1-b migrates at the rate predicted for a molecule of approximately twice the molecular weight of pub1. Such a species could be the product of disulfide-bond formation between two pub1 molecules or between pub1 and a protein of approximately the same molecular weight. In the absence of a hemagglutinin tag, no pub1 was detected (data not shown).

The association of ubiquitin with pub1 is refractile to both 1% LDS and 1 M formic acid, sensitive to both DTT and 0.1 M NaOH and co-incident with the appearance of pub1-a, an ~5–10 kDa DTT-sensitive higher molecular weight form of pub1. These observations suggest the presence of a thioester bond between pub1 and ubiquitin *in vivo*.

Discussion

It is becoming increasingly apparent that ubiquitin-dependent protein degradation plays a central role in cell cycle regulation. Cyclins are degraded by the ubiquitin system and the degradation of cyclin B is required to exit mitosis in *Xenopus* (Murray *et al.*, 1989). The metaphase to anaphase transition during mitosis appears to be initiated by sister chromatid separation, an event which seems to require ubiquitin-dependent degradation of an unknown protein (Holloway *et al.*, 1993). In *S.cerevisiae*, the degradation of the cdc28/clb inhibitor $p40^{SIC1}$ is required for transition from G_1 to S phase (Schwob *et al.*, 1994). Although $p40^{SIC1}$ has not been shown to be ubiquitinated, its degradation is dependent upon the ubiquitin-conjugating enzyme (E2) cdc34 (Schwob *et al.*, 1994). Similarly, FAR1, a protein required for pheromone-induced G_1 arrest in *S.cerevisiae* is degraded at the G_1/S transition in a cdc34-dependent fashion (McKinney *et al.*, 1993). Although some of the targets and E2s of the ubiquitin degradation system involved in cell cycle regulation have been identified, the protein ubiquitin ligases which determine the specificity of these reactions have not yet been cloned.

The level of cdc25 protein in fission yeast is tightly regulated by both transcriptional and post-transcriptional mechanisms (Ducommun *et al.*, 1990; Moreno *et al.*, 1990). In this report we have shown that cdc25 is ubiquitinated in *S.pombe* and we have cloned pub1, an E6-AP-related protein ubiquitin ligase involved in this process. Pub1 contains a region homologous to the putative catalytic domain of the human protein ubiquitin ligase E6-AP and forms a ubiquitin thioester *in vivo*. The *pub1* disruption post-transcriptionally increases the level of cdc25 protein *in vivo* behaving genetically like an activator of cdc25.

The simplest model which explains these observations is a direct ubiquitination of cdc25 by pub1 *in vivo*. Pub1-dependent ubiquitination may require one or more additional components. E6-AP for example, requires the oncogenic HPV E6 protein to both bind and ubiquitinate p53 (Huibregtse *et al.*, 1991, 1993b). In this regard it is interesting to note that pub1-b accumulates under the

same conditions and at the same time as does thioester-ubiquitinated pub1. Pub1-b may be composed of pub1 crosslinked by disulfide bond to a protein required for pub1-dependent ubiquitination. Assuming that pub1 directly ubiquitinates cdc25, the low level cdc25 ubiquitination observed in a *pub1*-disruption indicates the existence of a second cdc25 ubiquitination pathway. The ubiquitination of the *mat* α2 repressor in *S.cerevisiae* for example, involves two distinct pathways employing four different ubiquitin-conjugating enzymes (Chen *et al.*, 1993). Alternatively, pub1 may indirectly regulate cdc25 ubiquitination by, for example, targeting an inhibitor of cdc25 ubiquitination. While no direct inhibitors of ubiquitination reactions have been identified to date, cdc28/cln kinase activity in *S.cerevisiae* has been shown to inhibit degradation of the mitotic cyclin clb2 during G_1 (Amon *et al.*, 1994). It is possible that the increase of cdc25 protein levels observed in a *pub1* disruption may be due to an increase in translation of cdc25 as opposed to a decrease in its degradation. Given the overwhelming evidence that multiple ubiquitination serves as a general signal for proteolytic degradation, we feel this is a remote possibility (Finley and Chau, 1991; Ciechanover, 1994). However, the role of pub1 in the ubiquitination of cdc25 does not preclude it from regulating cdc25 at some other level, including translation.

Cdc25 is most probably not the only target of pub1. Disruption of *pub1* results in pleiotropic effects: the cells clump, are hypersensitive to certain nutrients and grow very poorly on certain carbon sources (our unpublished data). These phenotypes are not obviously attributable to cdc25 deregulation. In addition, overproduction of *pub1* is lethal in cells with the *cdc2-3w* mutation which do not require cdc25 function (our unpublished data). The presence of a CaLB domain in pub1 suggests that under conditions which raise intracellular Ca^{2+} levels, pub1 may target membrane-bound or -associated proteins. Interestingly, cdc25A has been shown to co-localize to the plasma membrane in mouse 3T3 cells (Galaktionov *et al.*, 1995a). We conducted a blast search of GenBank using either E6-AP or *pub1* as query sequence. Of the sequences recovered with homology to the putative catalytic domain of E6-AP, only pub1 and RSP5 had CaLB domains.

Pub1 represents a novel mechanism for cdc25 regulation. Loss of pub1 results in elevated levels of cdc25 protein and increased cdc25 activity *in vivo*. With this in mind it is intriguing to note that overexpression of *cdc25* may be oncogenic in humans. Overproduction of human cdc25A or cdc25B cooperates with either activated Ras or loss of Rb in mouse cells to form oncogenic foci (Galaktionov *et al.*, 1995b). Furthermore, 32% of primary breast tumors in humans were found to overexpress cdc25B (Galaktionov *et al.*, 1995b). If a functional homolog of *pub1* exists in humans, its loss may well prove oncogenic.

Materials and methods

Strains and media

All *S.pombe* strains were derived from the wild-type strains originally described by Leupold (1970). All strains used in this study are listed in Table I. *Schizosaccharomyces pombe* was grown in standard YE, YEA and PM media (Beach *et al.*, 1985) containing additional leucine as described at 150 μg/ml. Where indicated medium was supplemented with 20 μM thiamin. Standard *S.pombe* genetic procedures were followed (Gutz *et al.*, 1974).

Cloning sequencing and mapping pub1

A *S.pombe* cDNA library cloned into the *Not*I site of λZAP2 was immunologically screened with the anti-phosphotyrosine monoclonal antibody (UBI) (Lindberg *et al.*, 1988; Druker *et al.*, 1989; Lindberg and Pasquale, 1991). 2.5×10^5 plaques were screened and two putative positive clones were identified which were capable of generating anti-phosphotyrosine cross-reactive polypeptides upon induction in *Escherichia coli* as determined by Western blot analysis. *pub1* was one of these genes. The originally isolated *pub1* insert was used to re-screen the λZAP2 *S.pombe* cDNA library by hybridization to obtain a full-length cDNA. *In vitro* tyrosine kinase assays of pub1 lysates and purified fusion proteins failed to detect any tyrosine kinase activity (Wong and Goldberg, 1983; Braun *et al.*, 1984; Casnelli, 1991; Racker, 1991).

A series of unidirectional deletions of both the original *pub1* isolate and full length *pub1* cDNA was constructed in either pBluescript SK– or pBluescript KS– (Henikoff, 1987). Both strands of the cDNA clones were sequenced with a semi-automatic DNA sequencer (ABI 373A DNA sequencer). The predicted amino acid sequence was generated by an Intelligenetics program translation of the cDNA sequence. A blast search of GenBank was then performed to identify proteins with similar sequences. The *pub1* sequence was compared by visual inspection to the consensus sequence of the kinase catalytic domain (Hanks and Quinn, 1991).

We believe the cloning of *pub1* in a screen for tyrosine kinase activity was fortuitous. Although *E.coli* does not contain any tyrosine kinase activity, a phosphoamino acid analysis of total *E.coli* proteins will indicate the presence of phosphotyrosine. This phosphotyrosine is the product of acid hydrolysis of tyrosine-nucleotidylylated *E.coli* proteins (Foster *et al.*, 1989). We suspect a similar artifact resulted in the cloning of *pub1*. Depending upon the point of attack, hydrolysis of the nucleotidylyl-*O*-tyrosyl bond can yield two possible pairs of products; either a free tyrosine residue and a nucleotide monophosphate, or a phosphorylated tyrosine and a nucleoside. It has been shown that micrococcal nuclease can catalyze the hydrolysis of the adenylyl-*O*-tyrosyl moiety of *E.coli* tyrosine adenylylated glutamine synthetase *in vitro* producing a tyrosine phosphorylated protein (Kimura *et al.*, 1989). Expression of *pub1* in *E.coli* might increase either endogenous tyrosine nucleotidylylation or the rate of hydrolysis of these adducts. Either case could produce a sufficient amount of tyrosine-phosphorylated proteins to be detected with an anti-phosphotyrosine antibody in our screen.

The *pub1* gene was physically mapped by probing a collection of contiguous cosmid clones spanning the *S.pombe* genome with the 2.8 kb *pub1* cDNA (Mizukami *et al.*, 1993).

Gene disruption

A 948 bp *Sal*I–*Nsi*I fragment of the *pub1* cDNA was replaced with the 1.8 kb *ura4* gene. The resulting 3.7 kb disrupted cDNA was introduced into the diploid strain SP 826 and the Ura^+ transformants screened for inability to form single colonies in the presence of FOA. Genomic DNA from FOA-sensitive Ura^+ strains was then screened by Southern blot for disruption of a copy of the *pub1* gene. Colonies from one of these strains were screened by iodine staining for conversion of the mat locus from h^{+N}/h^{+N} to h^{90}/h^{+N}. Tetrads from this diploid strain were then dissected.

Southern and Northern blot analysis

Genomic *S.pombe* DNA was isolated from strains 972 and SP 1207, digested with *Eco*RI and Southern blot analysis was performed (Sambrook *et al.*, 1989; Moreno *et al.*, 1991). The 2.1 kb *Eco*RI fragment from the *pub1* cDNA was used as a probe.

Total *S.pombe* RNA was isolated as previously described (Moreno *et al.* 1991). Full-length *pub1*, *cdc25* and *ura4* were ^{32}P-labeled by random priming and used as probes as indicated in the text.

Epitope tagging of pub1

The *pub1* cDNA was used as a template for a PCR which introduced an *Nde*I site at the initiating methionine and a *Spe*I site immediately before the translational stop of the *pub1*. The *Nde*I–*Spe*I *pub1* fragment was then cloned into the *Nde*I–*Spe*I sites of the bacterial expression vector pET5C/HA.1 which contains a single hemagglutinin epitope bounded by an *Spe*I site on the 5′ end and a stop codon followed by a *Bam*HI site on the 3′ end. The resulting construct fused the C-terminus of pub1 to the 11 amino acid peptide TSYPYDVPDYA containing a

single hemagglutinin epitope preceded by the amino acids TS. The N-terminal *Nde*I–*Bam*HI fragment of this epitope-tagged *pub1* was cloned into the *Nde*I–*Bam*HI sites of the *S.pombe* expression vector pREP1 and the C-terminal *Bam*HI fragment was then cloned into the *Bam*HI site of the resulting construct. The final construct pREP1*pub1*HA consists of the *pub1* ORF with a C-terminal 11 amino acid extension containing the HA epitope cloned into the *Nde*I–*Bam*HI sites of pREP1.

Immunoprecipitation and immunoblotting of cdc25

Schizosaccharomyces pombe extracts for immunoblotting of cdc25 were prepared as previously described (Ducommun *et al.*, 1990). Cell pellets were resuspended in twice their volume of buffer I [50 mM Tris–HCl pH 8, 8 M urea, 1 mM EGTA, 5 mM EDTA, 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin and 10 μg/ml tosyl phenylalanine chloromethyl ketone (TPCK)]. The cells were lysed by vortexing in the presence of glass beads and the soluble proteins were recovered by centrifugation. Samples were immediately removed and boiled for 3 min in Laemmli sample buffer. The protein concentration of the lysate was determined by Bradford dye binding assay and Western blot analysis was performed as previously described (Bradford, 1976; Ausubel *et al.*, 1991). Blots containing untagged cdc25 were probed with an affinity purified anti-cdc25 polyclonal antibody B1 (Ducommun *et al.*, 1990). Blots containing hemagglutinin-tagged cdc25 were probed with 100 ng/ml 12CA5 anti-HA monoclonal antibody (Boehringer Mannheim). In both cases cdc25 was detected by ECL (Amersham).

To immunoprecipitate cdc25 we modified the previously described protocol (Ducommun *et al.*, 1990). Cell pellets were resuspended in 4 volumes of ice-cold buffer 2 (25 mM Tris–HCl pH 8.0, 60 mM β-glycerol phosphate, 15 mM *para*-nitrophenylphosphate, 0.1 mM ortho-vanadate and 0.1% Triton X-100) containing the same protease inhibitors as in buffer I. The cells were lysed by vortexing in the presence of glass beads at 4°C and the extracts immediately made 1% SDS and boiled for 3 min. The extracts were then diluted 10-fold in RIPA buffer lacking SDS and soluble proteins were recovered by centrifugation at 10 000 *g* for 15 min at 4°C. The protein concentration was then determined by Bradford assay (Bradford, 1976). The lysates were pre-incubated with protein A–agarose (Pierce) for 30 min and centrifuged at 10 000 *g* for 10 min both at 4°C. Immunoprecipitation was performed using affinity purified anti-cdc25 polyclonal antibody B1 and the immunoprecipitates was analyzed by Western blot for the presence of both ubiquitinated and hemagglutinin-tagged cdc25.

Immunoblot detection of ubiquitin

The same protocol was used to detect both free ubiquitin and ubiquitinated cdc25. The gel of interest was transferred to a sheet of 0.2 μm nitrocellulose (Schleicher and Schuell) which has been hydrated overnight in distilled water. After the transfer the nitrocellulose is incubated in distilled water at 100°C for 20 min and then Western blot analysis was performed as previously described (Swerdlow *et al.*, 1986; Ausubel *et al.*, 1991). All subsequent manipulations were done at room temperature. Blots containing ubiquitin were probed with an anti-ubiquitin polyclonal antibody (Sigma) and ubiquitin was detected by ECL.

Immunoblotting and immunoprecipitation of thioester-ubiquitinated pub1

Strain SP 1208 bearing either pREP1*pub1*HA or pREP1*pub1* was grown in minimal medium lacking thiamin for 10 h to a density of 10^7/ml after which cycloheximide was added to a final concentration of 100 μg/ml (Novak and Mitchison, 1987). Immediately before and 15, 30, 60 and 120 min after the addition of cycloheximide 50 ml aliquots were removed from the culture, the cells harvested by centrifugation, washed once with water and the cell pellets kept in a dry ice–ethanol bath until the end of the time course. The cell pellets were then thawed and resuspended in 3 volumes of cold lysis buffer (50 mM HEPES–NaOH pH 7, 1% lithium lauryl sulfate, 150 mM NaCl, 10 mM iodoacetamide, 5 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin and 10 μg/ml TPCK) (Hershko *et al.*, 1982; Haas *et al.*, 1985; Viestra *et al.*, 1985). The cells were lysed by vortexing in the presence of glass beads and the soluble proteins recovered by centrifugation at 10 000 *g* for 15 min at 4°C. The protein concentration was determined by Bradford assay (Bradford, 1976). Two gel samples of identical protein concentration were prepared from each time point one using standard Laemmli sample buffer and the other a modified Laemmli sample buffer which lacked β-me and had LDS in place of SDS. The standard Laemmli samples were subjected to electrophoresis in an 8% Laemmli gel at room temperature. The other samples were subjected to electrophoresis at 4°C in a modified Laemmli gel in which LDS is substituted for SDS (McGrath *et al.*, 1991). Both gels were transferred to nitrocellulose and subjected to Western blot analysis with the 12CA5 anti-HA monoclonal antibody as described above.

To immunoprecipitate thioester-ubiquitinated pub1, strain SP 1208 containing pREP1*pub1*HA or pREP1*pub1* was grown in minimal medium lacking thiamin at 30°C for 10 h to a density of 10^7/ml. The culture was then made 100 μg/ml cycloheximide and incubated for an additional hour at 30°C. Aliquots were removed immediately before and 1 h after the addition of cycloheximide. The cells were harvested, washed once with distilled water and kept in a dry ice–ethanol bath until lysis. Lysates were prepared in cold lysis buffer as described above. The lysates were diluted 10-fold with RIPA buffer containing the same protease inhibitors as the lysis buffer and lacking SDS. The soluble proteins were recovered by centrifugation and the protein concentration determined by Bradford assay. Thiol-ubiquitnated pub1 was immunoprecipitated from 5 mg of soluble lysate protein with the 12CA5 monoclonal antibody. The immunoprecipitates were then split into two equal aliquots. One aliquot was incubated for 1 h at 4°C in RIPA buffer containing both protease inhibitors present in buffer I and 20 mM DTT and then washed twice the same buffer. The other aliquot was treated identically with the same buffer lacking DTT. The beads were resuspended in Laemmli sample buffer and boiled for 3 min. Samples from both aliquots were run on both an 8% gel to detect pub1 and an 18% gel to detect free ubiquitin. Pub1 and ubiquitin Westerns were performed as described above.

Miscellaneous

Dapi (4,6-diamidino-2-phenylindole) staining of *S.pombe* cells was done according to Moreno *et al.* (1991).

pub1 was transcribed and translated *in vitro* using the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of ^{35}S Express (NEN).

Acknowledgements

We wish to thank Dr Hastie for his generous gift of the temperature-sensitive *mts2* allele and Dr Conklin for his generous gift of strain CFX 109-2R containing the triply-hemagglutinin-tagged cdc25 allele. In addition we would like to thank Dr Zhang for generously providing us with plasmid pET5C/HA.1 and Jim Duffy, Phil Renna and Mike Ockler for artistic assistance.

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Received on July 27, 1995; revised on September 29, 1995