Mutations in the bimC box of Cut7 indicate divergence of regulation within the bimC family of kinesin related proteins

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SUMMARY

Members of the bimC family of kinesin related proteins (KRPs) play vital roles in the formation and function of the mitotic spindle. Although they share little amino acid homology outside the highly conserved microtubule motor domain, several family members do contain a ‘bimC box’, a sequence motif around a p34cdc2 consensus phosphorylation site in their carboxy-terminal ‘tail’ region. One family member, Eg5, requires phosphorylation at this site for association with the mitotic spindle. We show that mutations in the Schizosaccharomycyes pombe cut7+ gene that change the bimC box p34cdc2 consensus phosphorylation site at position 1,011 and a neighbouring MAP kinase consensus phosphorylation site at position 1,020 to non-phosphorylatable residues did not affect the ability of S. pombe cut7+ genes to complement temperature sensitive cut7 mutants. Phosphorylation site mutants expressed as fusions to green fluorescent protein associated with the mitotic spindle with a localisation indistinguishable from similarly expressed wild-type Cut7. Cells in which cut7.T1011A replaced the genomic copy of cut7+ were viable and formed normal spindles. Deletion of the entire carboxy-terminal tail region did not affect the ability of Cut7 to associate with the mitotic spindle but did inhibit normal spindle formation. Thus, unlike Eg5, neither the p34cdc2 consensus phosphorylation site in the bimC box nor the entire tail region of Cut7 are required for association with the mitotic spindle.

Key words: Kinesin, Schizosaccharomycyes pombe, Cut7, Cell cycle, Mitosis

INTRODUCTION

In eukaryotic cell division the duplicated genome is segregated into the two daughter cells by the mitotic spindle. The spindle’s principle components are microtubules, which extend from microtubule organising centres (MTOC), one at each pole. The less dynamic (minus) end of the microtubules is found at the MTOC whilst the more dynamic (plus) end extends away from it (McIntosh and Hering, 1991). During spindle formation the MTOC nucleated arrays of microtubules interdigitate in an anti-parallel fashion to form the prophase spindle in which the two half-spindles are connected by motor mediated cross-linking of their anti-parallel microtubules. Spindle formation and function depends upon a balance of microtubule dynamics and the activities of MTOCs and a large number of motor proteins (Hyman and Karsenti, 1996; Waters and Salmon, 1997).

Many of the motor proteins required for mitotic spindle formation and function contain a region with high homology to the motor domain of kinesin heavy chain, the founder member of the kinesin family (Goodson et al., 1994; Moore and Endow, 1996). These kinesin related proteins (KRPs) have been grouped into families based on sequence similarities in the motor domains (Goodson et al., 1994; Moore and Endow, 1996). The families often contain proteins from different species that share more homology with each other than with other KRPs from the same species. Since the members of each family are generally involved in the same cellular processes it is sometimes assumed that such sequence similarities will always imply involvement in similar processes or even that the KRPs within a given family are functionally equivalent. It is possible, however, that with further characterisation the current families may be further divided into sub-families of molecules which are more closely related to each other, either by sequence or function, than to other family members.

Currently one of the best characterised of the KRP families is the bimC family which includes bimC (Aspergillus nidulans; Enos and Morris, 1990), Cut7 (S. pombe; Hagan and Yanagida, 1990), Cin8p and Kip1p (Saccharomyces cerevisiae; Hoyt et al., 1992; Roof et al., 1992); KLP61F (Drosophilia melanogaster; Heck et al., 1993), Eg5 (Xenopus laevis and Homo sapiens; Blangy et al., 1995; Le Guellec et al., 1991) and TKRP 125 (Nicotiana tabacum; Asada et al., 1997) as well as some partially sequenced genes such as KRP2 (Mus musculus; Sperry and Zhao, 1996). Loss of bimC KRP function causes the formation of abnormal spindles in which microtubules extend from two adjacent MTOCs of a monopolar spindle (Hoyt et al., 1992; Roof et al., 1992; I. Hagan, K. Tanaka and M. Yanagida, unpublished). Cin8p and Kip1p are also required for maintenance of the spindle once it
has formed (Saunders and Hoyt, 1992). Consistent with these results, the formation and maintenance of spindles in *Xenopus* egg extracts requires Eg5 (Savin et al., 1992) and injection of anti-Eg5 antibodies into cells causes a mitotic arrest with monopolar spindles (Blangy et al., 1995). Similarly antibodies against TKRP125 block translocation of phragmoplast microtubules in permeabilised plant cells (Asada et al., 1997). All of the bimC family kinesin related proteins localised to date have been shown to associate with the mitotic spindle (Asada et al., 1997; Barton et al., 1995; Hagan and Yanagida, 1992; Houliston et al., 1994; Hoyt et al., 1992; Roof et al., 1997).

Eg5 (Savin et al., 1992), KLP61F (Barton et al., 1995) and TKRP125 (Asada et al., 1997) all have plus end directed microtubule motor activity and a much slower rate of movement compared to kinesin heavy chain. This correlates with the high conservation of the motor domain between these KRPs and may signify a specialisation for roles in mitosis. The bimC family of KRPs share several invariant amino acids surrounding both the bimC box and an adjacent MAP kinase consensus phosphorylation site L/PxS/TP (Nigg, 1990) which surrounds both the bimC box and an adjacent MAP kinase consensus sites as a unit (Moore and Endow, 1996), all members of the bimC family KRPs have a central region predicted to form a coiled coil structure. This region in kinesin heavy chain has a structure consistent with a coiled coil (DeCuevas et al., 1992) and forms an extended stalk (Amos, 1987; Hirokawa et al., 1987; Hirokawa et al., 1993) which is also present in the bimC family member KRP130 (Kashina et al., 1996a,b). There is also a short ‘tail region’ at the carboxy terminus of kinesin heavy chain that is predicted to form a mixture of protein conformations suggesting a globular structure rather than a coiled coil (Hirokawa et al., 1989; Yang et al., 1989). A similar tail region exists in the bimC family KRPs. As with other KRPs there is virtually no homology between the bimC family members at the amino acid level outside the putative motors and it has been proposed that the diversity of sequence in the tails of different KRPs determines their individual function by binding to different cargos or substrates (Goldstein, 1993).

Given the sequence diversity of KRPs outside their motor domains it is particularly striking that most members of the bimC family of KRPs share several invariant amino acids surrounding a conserved p34<sup>cdc2</sup> kinase consensus phosphorylation site in their tail regions (Fig. 1), which has been termed the ‘bimC box’ (Heck et al., 1993). This is intriguing given that these proteins are essential for mitotic spindle formation and that p34<sup>cdc2</sup> kinase is the universal regulator of commitment to mitosis in eukaryotes (Nurse, 1990). Human Eg5 acts as a substrate for p34<sup>cdc2</sup> kinase in vitro and is phosphorylated on the same residue during mitosis in vivo (Blangy et al., 1995). Mutation of the threonine to alanine, which cannot be phosphorylated, prevents the association of both *Xenopus* and human Eg5 with the mitotic spindle (Blangy et al., 1995; Sawin and Mitchison, 1995); whilst substitution with serine, which can be phosphorylated, has no effect on the spindle association of *Xenopus* Eg5 (Sawin and Mitchison, 1995). These observations of Eg5 behaviour have been extrapolated to generate models for the potential role of phosphorylation in the regulation of bimC homologs in general (Walczak and Mitchison, 1996).

In addition to possessing the bimC box motif which includes the p34<sup>cdc2</sup> consensus phosphorylation site S/TpK/R (Nigg, 1993) found in other bimC KRPs, Cut7 also has a unique repeating motif (DxSLxx(L)ETTx(D/E); Hagan and Yanagida, 1990) which surrounds both the bimC box and an adjacent MAP kinase consensus phosphorylation site L/Pxs/TP (Nigg, 1993) which is also only present in Cut7 (Fig. 1). The presence of the bimC box and MAP kinase consensus sites as a unit interrupting the repeated motifs suggests that this short stretch...
containing the two kinase consensuses sites may be a significant regulatory region of Cut7.

We have investigated the role of the Cut7 bimC box p34ecd2 consensus phosphorylation site at amino acid 1,011 by mutation analysis. Mutations which prevent the spindle association of Xenopus and human Eg5 (Blangy et al., 1995; Sawin and Mitchison, 1995), did not affect the ability of Cut7 to associate with the mitotic spindle. Furthermore a cut7.T1011A strain was viable and the cells formed normal spindles. Our observations suggest that phosphorylation of threonine 1,011 is not required for mitotic spindle association or other essential Cut7 functions. We discuss the implications of these results for the prediction of KRP characteristics.

**MATERIALS AND METHODS**

**Yeast culture and genetic manipulations**

Strains used are listed in Table 1. Genetic manipulations and transformation of plasmids were by standard methods (Gutz et al., 1974; Moreno et al., 1991). Strains were grown in YES, EMMG (Moreno et al., 1991) or MSL (Egel et al., 1994) supplemented with 5 g l\(^{-1}\) arginine, 3 g l\(^{-1}\) potassium phosphate and 5 g l\(^{-1}\) Na\(_2\)HPo.\(_4\).\(_{12}\)H\(_2\)O. Appropriate supplements for each strain were added at 500 mg l\(^{-1}\).

**Sequencing of genomic DNA**

Genomic DNA was prepared from *S. pombe* cells using the Centra Systems Inc Puregene DNA isolation kit (Flowgen Instruments Ltd, Lichfield, UK) according to the manufacturer’s instructions apart from the addition of 1 mg ml\(^{-1}\) Zymolyase 100T (ICN Biomedicals Ltd, Thame, UK) to the cell suspension buffer. Fragments containing the regions of interest were PCR amplified using Taq polymerase (BCL, Lewes, UK) and the appropriate primers (see below). PCR products were purified using Qiaquick PCR purification columns (Qiagen Ltd, Crawley, UK) and sequenced by dye terminator cycle sequencing (Applied Biosystems, Warrington, UK) using the primers CAACCTGGCGATGCTTCTCTCAA and GACAGTTGAAATGGATC.

**Plasmid constructs**

DNA manipulations were by standard methods (Sambrook et al., 1989). Enzymes were purchased from New England Biolabs Ltd (Hitchin, UK).

**Creation of new unique sites in cut7**

New unique restriction sites were created in cut7\(^{+}\) by PCR mutagenesis. An EcoRI site was created by making a silent change in codon 887 which encodes serine from AGA to TCG, a non-coding region after the two stop codons. An Sall site by inserting GAGCTC 306 basepairs downstream of the new site. The following amino acid changes were created in this fragment: T1011A, S1020A, S1022A, T1024A (plasmids named as pks/cut7/T1011A for T1011A mutant). Following mutagenesis all mutants were checked by sequencing both strands of the entire fragment. The BamHI-EcoRI fragment from pks/cut7/10 was cloned into the BamHI and EcoRI sites of pks/cut7/T1011A and the other mutants to make the equivalent plasmids pBS/cut7/T1011A.

**Multicopy vectors**

The mutants were cloned into either pREP81 (Basi et al., 1993) or pREP41GFP (Griffiths et al., 1995) which contains green fluorescent protein (Cubitt et al., 1995) upstream and in frame with the NdeI site of the pREP41 polylinker. The NdeI fragment of pFP6 (Hagan and Yanagida, 1992), which encodes the amino terminus of Cut7 with an NdeI site at the start ATG of cut7\(^{+}\) was cloned into the NdeI site of pREP81 (Basi et al., 1993) and pGFP41 (Griffiths et al., 1995) to create pREP81/cut7/1 and pGFP41/cut7/1. A truncated version of pks/cut7/10 was created by removing the EcoRV fragment in the cut7\(^{+}\) coding region to create pks/cut7/d2 and the BamHI-EcoRI II fragment from pks/cut7/d2 was cloned into the BamHI and SmaI sites of pREP81/cut7/1 and pGFP41/cut7/1 to create pREP81/cut7/d2 and pGFP41/cut7/d2. The BamHI-SalI fragments of pbs/cut7\(^{+}\) and of the mutants such as pbs/cut7/T1011A were cloned into the BamHI and SalI sites of pREP81/cut7/d2 and pGFP41/cut7/d2 to create pREP81/cut7/T1011A and pGFP41/cut7/T1011A plus the other mutants. The size difference between the full length mutants and the truncated cut7/d2 simplified screening to ensure that the mutated versions of cut7\(^{+}\) had been cloned.

**Integrating vectors**

The integrating vector pINT5 (P. Wagner, K. Hill and J. R. Jenkins, unpublished) was modified using a linker to replace the BamHI site in the polylinker with a SacI site to create plNT6. The Xbal-SacI fragment of pREP81/cut7\(^{+}\) and pREP81/cut7/T1011A were cloned into the XbaI and SacI sites of pINT6 to create plNT8/cut7\(^{+}\) and plNT8/cut7/T1011A. An NdeI fragment encoding GFP (Griffiths et al., 1995) was cloned into pREP81 to create pREP81/GFP. The NcoI-SacI fragment of pGFP41/cut7/d2 (which encodes Cut7 with the central EcoRV fragment of cut7\(^{+}\) deleted) was cloned into the NcoI and SacI sites of pRP81/GFP to create p81GFP/cut7/d2. The XbaI-SalI fragment from this was cloned into the XbaI and SacI sites of plNT6 to create plN8TGF/cut7/d2. The NcoI-SalI fragments from pGFP41/cut7/T1011A and the other mutants were cloned into the NcoI and SacI sites of plN8TGF/cut7/d2 to create plN8TGF/cut7/T1011A plus the other mutants.

The NotI fragment of the pINT vectors encoding Cut7 was transformed into strain IHSP703 and integrants selected as ura\(^{+}\) leu\(^{-}\) transformants.

**Sequencing integrated vectors**

Following induction of integrated vector expression part of the culture was fixed and examined by immunofluorescence (see below). Genomic DNA was extracted from the remaining cells and amplified by PCR using the primers AATCCGAAGCAACGATTTCAG located within cut7\(^{+}\) and ATCGTAGGATGCCTGAA located in the vector sequence, ensuring that only DNA from the integrated vector and not the genomic copy of cut7\(^{+}\) was amplified. The amplified fragment was directly sequenced to screen for the presence of the expected amino acid changes.

**Multicopy PK Tag vectors**

The linker (TAAGAGCTCTTAA) which encodes a stop codon and an EcoRI site was ligated into the single EcoRV site of pks/cut7/d2 to create pks/cut7/RVS. The linker (ATCCATAGGTTAAGCTG) which encodes an ATG methionine start codon and an NdeI site was cloned into the EcoRV site of pks/cut7/d2 to create pks/cut7/RVN. This was cut with NdeI and ligated to create pks/cut7/RVN/2 and the EcoRV fragment from pks/cut7/10 inserted into the EcoRV site of pks/cut7/RVN/2 to create pks/cut7/RVN/3. The linker (AATAGAGCTCTTAA) which encodes a stop codon and an EcoRI site was ligated into the EcoRI
Table 1. Yeast strains

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Unless it is indicated otherwise, all strains were constructed in this study.

*Hirano et al., 1988
†Gutz et al., 1974
‡Hagan and Yanagida, 1992

A linker (TATCGAGCTCAG) which converts an NdeI to a SacI site was inserted into pREP81 to create pREP81/2. The SacI fragment from pREP42PK (K. Sheldrick and I. Hagan, unpublished) which includes a PK tag (Hanke et al., 1992) was cloned into the SacI site of pREP81/2 to create p81PK. The NdeI-EcoRIII fragments from pks/cut7/RIN and pks/cut7/RVN/3 were cloned into the NdeI and Smal sites of p81PK to create p81PK/cut7/RIN and p81PK/cut7/RVN.

The NdeI fragment from pFP6 (Hagan and Yanagida, 1992) encoding the amino terminus of cut7* was cloned into the NdeI site of p81PK to create p81PK/cut7/1. The BamHI-EcoRIII fragments of pks/cut7/10, pks/cut7/RIN and pks/cut7/RVN were cloned into the BamHI and Smal sites of p81PK/cut7/1 to create p81PK/cut7*, p81PK/cut7/RIN and p81PK/cut7/RVN.
RESULTS

Localisation of multicycopy GFP tagged Cut7

To investigate the function of the bimC box p34\(^{cd2}\) consensus phosphorylation site in Cut7 we first established a suitable system in which to assay the behaviour of the mutant proteins. Full length Cut7 was expressed as a fusion with green fluorescent protein GFP (Cubitt et al., 1995) at the amino terminus of Cut7 from an inducible promoter in a multicycopy vector (Griffiths et al., 1995). Localisation of the tagged mutant protein could therefore be distinguished from the native protein encoded by the genomic copy of cut7\(^{+}\). This approach has been effective in testing Eg5 mutants, where multicycopy tagged mutant proteins failed to localise to the spindle in cells containing wild-type Eg5 (Blangy et al., 1995; Sawin and Mitchison, 1995). Multicycopy vector based expression produces variable protein levels within the different cells of a population (Hagan and Yanagida, 1992; Olsson et al., 1993). As only 10\% of wild-type cells are in mitosis at any one time it is impractical to screen a wild-type population using multicycopy cloning vector based expression of GFP Cut7 fusion proteins as very few cells would be both in mitosis and contain sufficient GFP-fusion protein to produce a detectable signal. We therefore enriched for mitotic cells by using a mutation which arrests at metaphase of mitosis, nuc2.663 (Hirano et al., 1988). We have previously shown that this metaphase arrest arising from mutation of this cyclosome sub-unit results in a Cut7 distribution which is indistinguishable from the distribution of Cut7 in wild-type cells containing mitotic spindles of the same length (Hagan and Yanagida, 1992). The GFP fluorescence signal on the spindles resembled the staining observed with antibodies to the endogenous wild-type Cut7 protein in nuc2.663 cells at the restrictive temperature (Fig. 2A) (Hagan and Yanagida, 1992).

Localisation of mutant GFP Cut7 fusions

To test whether the p34\(^{cd2}\) consensus phosphorylation site in the tail region was essential for Cut7 spindle association a series of mutants were created by site directed mutagenesis. These changed threonine 1,011 of the p34 cdc2 consensus phosphorylation site to the non-phosphorylatable residues alanine, glutamine and asparagine. Threonine 1,011 was also mutated to the charged residues aspartic acid or glutamic acid which, in some cases, may mimic the presence of a phosphorylated amino acid. All mutant proteins localised to the spindle with the same distribution as the wild-type GFP-Cut7 fusion (Fig. 2B,C,E,G,I). Only the mutation T1011N (Fig. 2E) was different in that very few cells contained a GFP signal, but in those containing fluorescence the distribution was identical to wild type.

Function of multi-copy GFP tagged Cut7

Although the GFP tagged Cut7 could associate with the mitotic spindle it was possible that it was non-functional. Multicycopy GFP tagged Cut7 was expressed in strains containing the temperature sensitive alleles cut7.322, cut7.22 and cut7.24 (Hagan and Yanagida, 1990, 1992) and in all cases could complement the temperature sensitivity of the cut7\(^{+}\) alleles (data not shown) including the recessive allele cut7.24 (Hagan and Yanagida, 1990, 1992; I. Hagan and M. Yanagida, unpublished). Since GFP tagged wild-type Cut7 appeared to function normally when expressed as a multicycopy gene, we
used it to compare the localisation of Cut7 at the spindle poles of nuc2.663 cells with that of Sad1 (Hagan and Yanagida, 1995) which has been shown by immunogold labelling with anti-Sad1 antibodies to be an SPB component (I. Hagan and N. Hajibagheri, unpublished). Raw data images were processed into high contrast false colour images so as to emphasise the peak intensity of the fluorescent signals at the expense of the weaker signal coming from the spindle. When super-imposed these high contrast images showed that the main localisation of the GFP Cut7+ fluorescence was nearer the centre of the spindle than that of the anti-Sad1 immunofluorescence (Fig. 2K). A similar localisation was also found with the T1011A mutant (Fig. 2L).

**Function of multi-copy cut7 mutants**

It was possible that the presence of the GFP fusion protein might be affecting the function of Cut7 and suppressing the effect of any of the mutations in the tail. The mutants were therefore expressed as full length untagged proteins from multicopy vectors with a lower strength promoter in strains containing either the cut7.322, cut7.22 or the recessive cut7.24 temperature sensitive alleles of cut7+. All complemented the temperature sensitive alleles and supported colony formation on plates at the restrictive temperature indicating that all of the mutants are able to complement cut7+ mutants when expressed from multiple copies (data not shown).

**Expression of single copy GFP tagged cut7+**

One potential problem with these experiments was the possibility of recombination between the cut7 sequences on the multicopy plasmids and the genomic copy of cut7+. This could produce multicopy versions of cut7 that had lost the p34cdc2 consensus site mutation and regained a wild-type bimC box. If any of the mutants being tested had a deleterious effect on cell growth then there would be a positive selection for such a conversion event to take place during colony formation before we could analyse the strain. Some mutant cut7 genes were therefore integrated into the genome under the control of a weak inducible promoter. The validity of this approach was demonstrated by the ability of an integrated GFP cut7+ gene to complement the recessive temperature sensitive allele cut7.24 (Fig. 3).
Location of GFP Cut7+ fusion was followed in living cells by fluorescence microscopy for 18-20 hours after induction until GFP fluorescence was observed, whereupon the culture was fixed and examined by fluorescence microscopy. The level of protein present caused even staining of the entire anaphase spindle with none of the distinct localization features previously found using anti-Cut7 antibodies in immunofluorescence staining (Hagan and Yanagida, 1992) (data not shown). Since such an even staining pattern had been observed by immunofluorescence with over-expressed cut7+ (Hagan and Yanagida, 1992) this suggested that the expression level required to see autonomous GFP fluorescence was too high. Shorter induction times reduced the fluorescence signal below the detection threshold. We therefore used polyclonal anti-GFP antibodies to amplify the signal of GFP tagged Cut7 so that lower protein levels could be detected. With antibody staining, GFP Cut7+ was now detectable on the mitotic spindle following shorter induction times (10-12 hours) and its distribution closely resembled the staining found using anti-Cut7 antibodies (Fig. 4) (Hagan and Yanagida, 1992). Short spindles stained along their entire length with a marked concentration towards their poles (Fig. 4A, cell 1). A punctate pattern was present in longer spindles (Fig. 4B, cell 2) and the protein concentrated in the centre of longer anaphase spindles (Fig. 4C, cell 3). In cells with longer spindles where GFP Cut7 stained the central spindle region only a few cells such as the example in the inset panel of Fig. 4C also had staining towards...
the spindle poles. This contrasts with anti-Cut7 staining where there was staining at or adjacent to the spindle poles throughout mitosis (Hagan and Yanagida, 1992). This difference might arise either from a genuine failure of the GFP tagged Cut7 to localise to this region to the same extent as the non-tagged protein in the later stages of mitosis, or as a result of epitope masking of the GFP at these stages, or because the precise level of induction is important to detect the localisation at the spindle poles. Differences in epitope accessibility are possible since GFP is located at the amino terminus of Cut7 whereas the polyclonal anti-Cut7 antibodies were raised against the carboxy-terminal half of the molecule (Hagan and Yanagida, 1992). Similar patterns to the GFP Cut7 fusion distribution were also observed with versions of Cut7 containing an amino-terminal epitope tag (see below).

**Localisation of GFP tagged single copy mutants of Cut7**

Induction and localisation of GFP Cut7 mutant proteins T1011A, T1011D and T1011E expressed from integrated single copy genes in strains containing a genomic cut7+ gene revealed a pattern which was indistinguishable from that of GFP Cut7+ (compare Fig. 5C,D,F and Fig. 4). To ensure that no gene conversion had occurred during integration cells were removed at the end of the experiment and the fragment corresponding to amino acids 888-1,085 of the integrated GFP cut7 gene was sequenced. All sequences still contained the expected mutations (data not shown).

**Function of GFP tagged single copy mutants of Cut7**

Wild-type and mutant GFP Cut7 expressed in a cut7.24 strain could complement the temperature sensitivity of this allele (Fig. 3). As it was possible that the presence of GFP in the fusion protein was suppressing any deleterious effects caused by the mutations on protein function, integrated cut7+ and Cut7.T1011A which did not contain the GFP tag were also tested for complementation. Both were indistinguishable from GFP cut7+ (Fig. 3). Thus the presence of the mutation in the carboxy terminus did not affect the ability of Cut7 to complement cut7.24.

**Function of a genomic allele cut7.T1011A**

It was possible that cut7+, cut7.24, cut7.22 or cut7.322 genes were able to complement the mutants by the formation of a functional multimeric protein structure containing both a mutant and wild-type or temperature sensitive Cut7. We therefore examined the ability of the mutant Cut7.T1011A to function in the complete absence of any other Cut7 protein by replacing the genomic copy of cut7+ with the cut7.T1011A gene. Two independent cut7.T1011A integrants formed apparently normal mitotic spindles (Fig. 6). The ability of Cut7.T1011A to associate with the spindle was verified by staining with anti-Cut7 antibodies (data not shown). Only the presence of a small number of anucleate cells (0.4 %) suggested any form of mitotic defect.

If phosphorylation of threonine 1,011 does have a role in the regulation of Cut7 activity then it must be very subtle under...
normal growth conditions. It could, however, become essential under other circumstances. We therefore assessed cut7.T1011A strains for thiabendazole (an anti-microtubule drug), heat (36°C) and cold (20°C) sensitivity but found no obvious differences in comparisons with an isogenic cut7+ strain (data not shown). We also examined spore formation in a strain containing cut7.T1011A. Only a slight increase in the formation of defective asci containing only 2 or 3 spores was detected indicating that there was no strong meiotic defect (data not shown). Thus Cut7.T1011A is virtually indistinguishable from wild-type Cut7 in all assays tested.

Mutation of other potential phosphorylation sites
The bimC box lies in a region of Cut7 which interrupts three copies of a repeating motif not present in other bimC family KRP (Fig. 1B). This may indicate that the region between the repeats is the target for regulatory modifications. Since we failed to detect any significant effect of mutations in the bimC box p34cdc2 consensus phosphorylation site we mutated other sites within the region delineated by the repeating motif. These changes were mutation S1013A, mutation of the MAP kinase consensus phosphorylation site at position 1020 either alone (T1020A), or in combination with the p34cdc2 consensus phosphorylation site (T1011A, T1020A), or in combination with nearby threonine and serine residues (T1020A, S1022A, T1024A). All of these mutants associated with the mitotic spindle when expressed as multicopy GFP fusions in a cut7+ integrant of the Cut7 888-1,085 disruption at the ura4+ locus. In contrast, from amino acids 111-1,085, 9 integrants at the ura4+ locus were obtained from 76 stable transformants. If 9/76 (0.12) is the normal integration frequency at the cut7+ locus, the probability of not finding an integrant of the Cut7 888-1,085 disruption at the cut7+ locus amongst 39 integrants is 0.8839 (0.007). This suggested that there might be some selection against a disruption of the tail alone, even in a diploid strain that still contained a wild-type copy of the cut7+ gene.

To test which regions of Cut7 might be required for its function a series of truncations tagged with the SV5 ‘PK’ epitope recognised by the monoclonal antibody mAb336 (Hanke et al., 1992; Randall and Young, 1988) were created in a multicopy vector under the control of an inducible promoter (K. Sheldrick and I. Hagan unpublished) (Fig. 7). Neither Cut7-HST, Cut7-ST, Cut7-H, Cut7-T nor Cut7-HS caused any obvious effect on colony formation when expressed in a cut7+ strain (data not shown). However in a non-tagged form Cut7-HS caused a slight retardation in colony formation compared to control strains. When induced in a cut7+ strain growing in liquid culture Cut7-H and Cut7-T caused no obvious defects in spindle formation whilst Cut7-ST caused a slight proportion of V shaped monopolar spindles (0.8%). The most dramatic effect was caused by Cut7-HS where, following induction, 29.6% of cells had V shaped monopolar spindles (Fig. 8B) and an additional 30.0% of cells were anucleate indicating a strong mitotic defect. This strong dominant effect might explain our earlier failure to produce the identical disruptions of the genomic Cut7 and our previous inability to get transformants using a similar construct from which a truncated version was constitutively expressed (Hagan and Yanagida, 1990).

The apparent discrepancy between the magnitude of this effect of Cut7-HS and the ability of the strain to form colonies on plates might be explained by the much longer time required for colony formation. This might permit selection of cells...
containing fewer copies of the plasmid encoding the Cut7 truncation, thus reducing the apparent severity of any effect of the mutation.

As the nuclear envelope does not break down during mitosis in *S. pombe* it was possible that some of the truncated proteins might not be able to enter the nucleus. We therefore examined the distribution of the PK tagged proteins. Whilst Cut7-HST entered the nucleus and associated with the spindle in a manner indistinguishable from the GFP tagged Cut7, Cut7-ST, Cut7-H and Cut7-T were all cytoplasmic. Therefore the lack of any significant phenotype in these cases may be due to the failure of these proteins to enter the nucleus, making conclusions about their function difficult. Cut7-HS not only entered the nucleus but associated with the abnormal monopolar spindles (Fig. 8D).

**DISCUSSION**

We have examined the role of a potential p34^cdk2^ phosphorylation site in the bimC box in the carboxy-terminal tail of Cut7 which, together with surrounding amino acids, is conserved in many bimC like KRP. Phosphorylation at this site is required for the association of both human and *Xenopus* Eg5 with the mitotic spindle (Blangy et al., 1995; Sawin and Mitchison, 1995). In contrast, mutation of this site in Cut7 to non-phosphorylatable residues did not affect the association of Cut7 with the mitotic spindle or its localisation on the spindle. We also examined the effect of substituting the acidic residues glutamic acid and aspartic acid, which could potentially mimic the phosphorylated state, and again found no effect on spindle association. Although Eg5 requires a phosphorylated residue at the equivalent location for spindle association, mutation of threonine to glutamic acid in *Xenopus* Eg5 (Sawin and Mitchison, 1995) and aspartic acid in human Eg5 (Blangy et al., 1995) both prevented association of Eg5 with the mitotic spindle. Again the behaviour of Cut7 mutant protein differs from that of the Eg5 mutants.

Even when the genomic copy of cut7^+^ was replaced by cut7.T1011A, the only difference detected was in the number of anucleate cells, which rose from 0 to 0.4%. Thus, contrary to predictions based on the sequence homology of the bimC box surrounding this site, this region does not appear to act in Cut7 in the same way as in Eg5 to regulate association of the protein with the mitotic spindle. It is possible that the function of this site in Cut7 only becomes critical under some circumstances; however, we have no evidence at present that the very minor changes detected are not simply due to replacement of the threonine at this position with another amino acid, as one might expect with any random change in
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this region, rather than an effect caused by lack of phosphorylation. These results raise the possibility that this site may not be phosphorylated at all in Cut7.

The different behaviour of Cut7 and Eg5 bimC box mutants may arise from the sequence divergence at the end of their bimC boxes. Most bimC KRPs contain a YPSxL consensus at the end of the bimC box which has been replaced by a MAP kinase consensus sequence in Cut7. It is possible that this part of the bimC box corresponds to a second regulatory target. We therefore simultaneously mutated the threonine at the MAP kinase consensus phosphorylation site plus the adjacent serine and threonine residues to alanine. This mutant could still associate with the mitotic spindle. Even mutation of the MAP kinase consensus site either alone or in combination with the p34cdc2 consensus site did not prevent association of Cut7 with the mitotic spindle in the same way as similarly tagged wild-type Cut7.

One possible conclusion from the point mutagenesis was that the carboxy-terminal tail region was not required for Cut7 function. However, deletion of this entire region caused a dominant effect with the formation of monopolar spindles and the accumulation of anucleate cells, indicating that the tail region is required for normal Cut7 function. No such dominant effect was noted in tissue culture cells with a ‘tail-less’ truncation of Xenopus (Sawin and Mitchison, 1995) or human Eg5 (Blangy et al., 1995). Furthermore, neither of the Eg5 truncations were associated with the mitotic spindle whilst the Cut7 truncation was found on the defective spindles. Again this emphasises the difference in the regulation of Cut7 and Eg5 since the presence of the tail is not required for the association of Cut7 with the spindle microtubules.

It would seem surprising if the p34 cdc2 consensus site had no function at all in Cut7 given the level of conservation between this region in Cut7 and other bimC family KRPs. It is possible that the regulation of the different bimC KRPs has diverged as a result of differences in mitosis between different species. Thus in Xenopus and human cells where nuclear envelope breakdown accompanies the start of mitosis, this may require a specific regulation of Eg5 association with the spindle to co-ordinate its activity with the other events of mitotic spindle formation. Alternatively, the regulation may be required to ensure that cytoplasmic Eg5 is unable to associate with the microtubules of the interphase cytoskeleton and disrupt its structure or inhibit its disassembly at the start of mitosis. S. pombe has a closed mitosis during which the nuclear envelope remains intact. As Cut7 is a nuclear protein throughout the cell cycle (Fig. 4) (Hagan and Yanagida, 1992) budding yeast does not face similar problems. The bimC family KRPs Cin8p and Kip1p which lack this p34cdc2 consensus site are found in S. cerevisiae which also has a closed mitosis. However, it is unclear if the absence of a bimC box results from the closed mitosis or the differences in the timing of mitotic events between S. cerevisiae and other organisms (Forsburg and Nurse, 1991; Nurse, 1985).

**Fig. 8.** Localisation of Cut7-HS truncation. A cut7+ strain containing the plasmid p81PK/cut7/RIS which encodes the PK tagged Cut7-HS truncation under the control of a weak inducible nmt1+ promoter was grown at 32°C for 16 hours in EMMG containing either 2 μM thiamine to repress expression of the plasmid encoded the Cut7 truncation (A,C), or with no thiamine (B,D) to induce expression of the Cut7 truncation. Cells were fixed stained with antibody and examined by immunofluorescence. Each panel is a composite. (A and B) Three images with immunofluorescence following staining with TAT1 anti-α tubulin antibody in the upper image, immunofluorescence with anti-Sad1 antibodies in the centre panel and a phase contrast image with DAPI stained chromatin in the lower panel. (C and D) Two images with immunofluorescence following staining with mAb 336 (anti-PK tag antibody) in the upper image and a phase contrast image with DAPI stained chromatin in the lower panel. Scale bar, 10 μm.

**Concluding remarks**

Our data suggests that it is unlikely that phosphorylation at the potential p34cdc2 kinase consensus phosphorylation site that forms the core of the bimC box conserved region in the tail of Cut7 is required for the association of Cut7 with the mitotic spindle. Furthermore, Cut7 can still associate with the mitotic spindle even after deletion of the entire tail region. This behaviour contrasts with that of human and Xenopus Eg5 in
REFERENCES


