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Domains of p85^{cdc10} required for function of the fission yeast DSC-1 factor

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ABSTRACT

p85^{cdc10} is a component of the *S.pombe* DSC-1 complex, which is thought to mediate periodic transcription of genes in late G1. In order to understand the role of p85^{cdc10} in the function of this complex, we have analysed which domains of p85^{cdc10} are required for biological activity and the formation of a stable DSC-1 complex *in vitro*, both in *cdc10* temperature sensitive and null backgrounds. No DSC-1 activity is found in the absence of p85^{cdc10} and the activity of the complex is reduced or absent in all *cdc10^{ts}* mutants tested. Full biological activity and rescue of a *cdc10::ura4$^+$* null allele requires the N-terminal domain, the *cdc10/SWI6* repeats and the helical C-terminal region. In the absence of p85^{cdc10}, both the C-terminal and *cdc10/SWI6* repeat domains are required for DSC-1 activity *in vitro*. In a *cdc10^{ts}* background, rescue of DSC-1 activity and complementation of mutants, requires only expression of the C-terminal domain, though the presence of the *cdc10/SWI6* motifs enhances its activity. The N-terminal domain, alone, or in combination with the *cdc10/SWI6* motifs, does not have biological activity, and does not restore DSC-1 activity. We conclude that both the C-terminal domain of p85^{cdc10} is critical for formation of the DSC-1 complex and that the *cdc10/SWI6* motifs also play a role, perhaps by stabilizing the complex. Our data also suggest that the *S.pombe* DSC-1 complex contains more than one molecule of p85^{cdc10}.

INTRODUCTION

The control point in late G1 at which the yeast cell assesses its environment is called 'start' (1, 2). If conditions are appropriate, cells traverse start and become committed to the mitotic cell cycle. During exponential growth of *S.pombe* cells in rich media, the start control is cryptic and the G2, or mitotic, control is rate limiting for cell cycle progression. However, in small cells such as those produced by outgrowth of spores, nutrient limitation or *wee* mutations, the size control upon the initiation of DNA synthesis becomes rate limiting for cell cycle progression (3–5).

The definition of genes required for the traverse of start is an operational one, relying on the differential ability of cells to conjugate or sporulate before and after the traverse of start (1, 6–9). A screen of *S.pombe* cell cycle mutants has shown that *cdc2*, *cdc10* and *sct1/res1* functions must be executed for the traverse of start (8, 10, 11).

The *sct1* gene (also called *res1*; 11) was identified as a suppressor which bypasses the requirement for p85^{cdc10} (11, 12), and as a multicopy suppressor of mutations in the *ran1* gene (10). It is an essential gene at high and low temperatures, though at an intermediate temperature cells are viable (10). Overexpression of *sct1* will rescue a *cdc10::ura4$^+$* null allele, but not *vice-versa*.

The p34^{cdc2}/*CDC28*p kinase is involved in the traverse of both of the major control points in the yeast cell cycle, being independently required both for the start and for the mitotic controls (1, 8, 13–16). Studies of the execution points of *cdc2* and *cdc10* in *wee* mutants have suggested that *cdc2* acts upstream of *cdc10* (17).

The *cdc10* gene is essential for cell division (12). Execution of its function is not regulated at the level of transcription (18), or changes in p85^{cdc10} protein level (19), either during progression through the cell cycle or upon exit from it into stationary phase. p85^{cdc10} has most homology with *S.pombe sct1/res1* (10, 11) and the products of *SWI4* and *SWI6* of *S.cerevisiae*, which are components of the SBF transcription factor that regulates G1 specific expression of genes such as *HO* and the G1 cyclins (20–23).

p85^{cdc10} also shows similarity with a number of other proteins *via* a 33 amino acid repeat known as the *SWI6/cdc10* box or ankyrin repeat (24). Genetic evidence has shown that these repeats are functionally important in the p85^{cdc10}, *glp1* and *lin12* proteins (25–27). The role of this motif in p85^{cdc10} is unclear, though it has been suggested that it mediates interactions with other proteins in the case of ankyrin (28), GA binding protein (29) and in the regulation of nuclear localisation of *dorsal* by *cactus* (30) and NFκB by IκB (31).

After traverse of start, progress to S-phase is accompanied by an increase in the expression of histones and some or all of the genes required for DNA synthesis. In *S. cerevisiae*, the coordinate expression of DNA synthesis genes is dependent upon a cis-acting sequence element called the MCB (MluI Cell Cycle Box; 32).

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A transcription factor called DSC-1 (DNA synthesis control), comprised of at least the products of the *SWI6* gene and a 120kD protein, binds specifically to these elements and is thought to mediate the cell cycle regulated transcription (33–35). Mutations in *SWI6* abolish both the activity of the DSC1 transcription complex and the periodic expression of the DNA synthesis genes (34, 35). UV crosslinking experiments suggest that the 120 kDa protein, rather than *SWI6*p is the primary DNA binding component of the complex (35). The activity of *CDC28*p kinase is also required for activation of G1 specific transcription (36, 37).

In *S.pombe*, the transcripts of most genes required for S-phase do not increase in abundance after traverse of start. However, a few genes, such as *cdc22* (38, 39) do show a significant increase in late G1. A DSC-1 like factor has been identified which binds to the promoter of the *cdc22* gene (40). This region contains multiple MCB elements which have been demonstrated to confer periodic expression to a reporter gene in *S.pombe* (40).

Both $p85^{cdc10}$ and $p72^{sct1}$ are components of the *S.pombe* DSC-1 like complex (11, 40). The DNA binding activity of the complex is thermolabile in *cdc10* thermosensitive mutants and can be supershifted by antibodies to $p85^{cdc10}$ (40) or $p72^{sct1}$ (11). Execution of *cdc10* function is also necessary for the periodic accumulation of histone transcripts in S-phase (41), though it is not known if this effect is direct.

In this study, we demonstrate that the integrity of the C-terminal domain and the *cdc10/SWI6* repeats is required for the formation of the *S.pombe* DSC-1 like complex. It is likely that this complex contains more than one molecule of $p85^{cdc10}$.

METHODS

Strains and genetic techniques

The growth media, genetic and physiological procedures used for fission yeast have been described (3, 42, 43). The media used were Yeast extract (YE), containing 50 μg/ml each of adenine, uracil, leucine, lysine and histidine, Malt Extract (ME), and EMM2 minimal, supplemented as required. To aid identification of diploids and mutants, Phloxin B (5 μg/ml) was added to media when required. The strains used in this study have been described (12, 13). *S.pombe* was transformed by the lithium acetate method (44).

For assays of biological function of $p85^{cdc10}$ fragments, the *cdc10V50*, *cdc10-129*, *cdc10M47* and *cdc10MBC4* alleles were tested; to assess whether fragments of $p85^{cdc10}$ were capable of rescuing a *cdc10::ura4*$^+$ null allele, plasmids were introduced into the strain SP767 (Marks et al., 1992; *ade6M210/ade6M210 leu1-32/leu1-32 ura4D18/ura4D18 cdc10*$^+$*/cdc10::ura4*$^+$ h^{+N}/h^{90}), selecting for leucine prototrophy. The colonies were allowed to undergo meiosis, and spores were plated onto media selective for leucine and uracil prototrophy.

Microscopy and antisera

Antisera to full length $p85^{cdc10}$, expressed in bacteria, and the TAT-1 monoclonal antibody to tubulin have been described (12, 45). In western blotting experiments, bound antibodies were revealed using alkaline phosphatase conjugated secondary antibodies (Promega). For immunofluorescence of $p85^{cdc10}$, cells were fixed in 3% formaldehyde for 15 minutes and processed as described (46). The specificity of the antibody used has been previously defined by western blotting (12); no signal is seen in *cdc10::ura4*$^+$ *sct1-1* cell extracts, which lack $p85^{cdc10}$. To confirm the specificity of the immunofluorescence, *cdc10::ura4*$^+$ *sct1-1* cells were fixed as described above and incubated with the antibodies to $p85^{cdc10}$ or tubulin. The tubulin signal was present, indicating that cells had been adequately permeabilised and subcellular structures had been adequately preserved, the nuclear signal for $p85^{cdc10}$ was absent, confirming the specificity of the serum used (A.R., J. Marks, and V.S., EMBO J, submitted).

Protein extraction and bandshift assays

Exponentially growing cells were harvested, washed once with ice cold distilled water, resuspended in 50 mM Tris–HCl, pH 7.5, 50mM KCl, 0.1% Triton X-100, 25% glycerol, 2mM DTT, 0.2mM PMSF containing 25μg/ml each chymostatin, leupeptin, antipain, aprotinin and pepstatin A (all Sigma), and broken by vortexing with acid washed beads. Supernatants were clarified by centrifugation for 15 minutes in an eppendorf microcentrifuge at 4°C. Protein estimation was performed using the Bio-Rad Protein Assay kit. Bandshifts were performed as described (40), using a 132bp fragment from the promoter of the *cdc22* gene as probe. Binding reactions contained 1ng of ^{32}P end-labelled probe and 30 μg of total clarified protein extract in 75 mM Tris–HCl, pH 7.5, 75mM KCl, 25mM $MgCl_2$, 37.5% glycerol, 2mM DTT, 0.5mM PMSF and 37.5μg/ml each chymostatin, leupeptin, antipain, pepstatin A and aprotinin (final concentration, all Sigma). Poly dI/dC (Boehringer Mannheim) and single stranded *E.coli* DNA were used as nonspecific competitors at 0.1 and 0.5 μg/ml, respectively. Binding reactions were analysed by electrophoresis through a 4% acrylamide (19:1 acrylamide: bis-acrylamide) gel at 100 V. for 6 hours in 0.5×TBE, except where indicated otherwise.

Molecular techniques

Standard procedures were used for all DNA manipulations (47). The plasmids used in this study are shown in figure 2. They are based on either pDW232 (48), or pREP3 (49), modified to delete the initiating methionine from the polylinker, and express the following fragments of the *cdc10* gene, numbered according to the published sequence (18).

p4.1: 4.1 kb *Hin*dIII fragment from *cdc10* locus (18).

p2.45: *cdc10* gene with *Nco*I and *Bam*HI sites introduced at the ATG (position 149; 12).

p1.65 is the *Stu*I–*Hin*dIII fragment from residues 996 to 2606.

p1.44 is the *Hae*II–*Hin*dIII fragment from residues 1158 to 2606.

p1.27 is the *Ase*I–*Hin*dIII fragment from residues 1328 to 2606.

p1.15 is the *Pst*I to *Hin*dIII fragment from residues 1459 to 2606.

pN19 and **pE1** result from a exoIII deletion series made on plasmid p1.15 and run from nucleotides 1640 and 1737, respectively to the *Hin*dIII site at 2606.

pE1 I545 is identical with pE1 except that the initiating methionine at position 545 was mutagenised to Ile using the primer GTTTCCCTTATTAGTGAA and the Muta-Gene kit (Bio-Rad).

p2.20 was constructed by insertion of a linker TTAATTAATTAA into the *Xho*I site of p2.45 at residue 2335, truncating the C-terminal 38 amino acids of $p85^{cdc10}$.

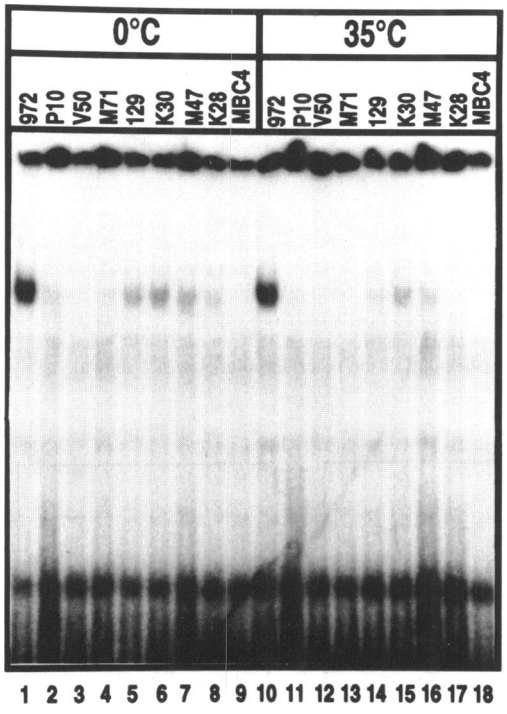

Figure 1. DSC-1 like bandshift activity is thermolabile in *cdc10*^{ts} mutants. Gel retardation assays on extracts from either wild type or the indicated *cdc10*^{ts} mutants, which represent all the 8 alleles (27), were performed either before (lanes 1−9), or after (lanes 10−18) preincubation of the extracts for 5 minutes at 35°C. Longer exposures of the gel showed small amounts of DSC-1 activity in *cdc10V50* but not *cdc10MBC4* (not shown).

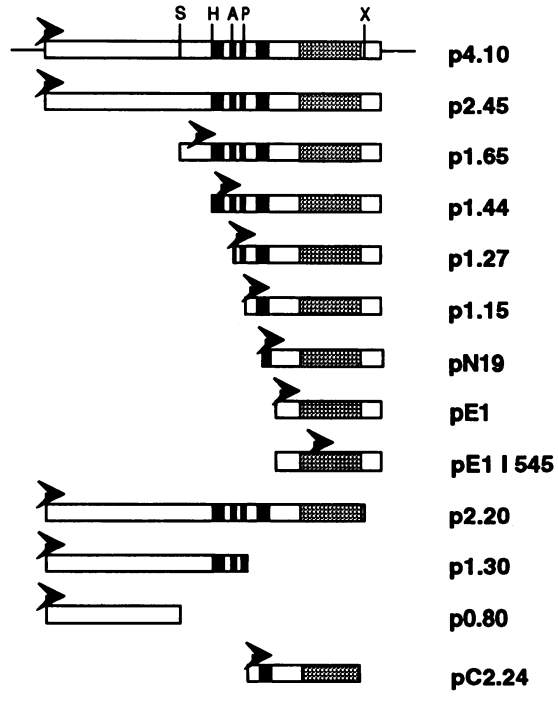

Figure 2. Structure of plasmids expressing fragments of p85^{cdc10}. Maps of *cdc10* fragments used in this study. The solid black boxes indicate the two complete and two partial copies of the *cdc10/SWI6* repeat (27), while the grey hatched region corresponds to the helical domain. The arrows indicate the position of the first methionine for translation. Western blots showed that in all cases, proteins of the expected size were produced in cells (not shown). Restriction site used in the constructions are indicated as follows: S, *Stu*I; H, *Hae*II; A, *Ase*I; P, *Pst*I; X, *Xho*I.

p1.3 and **p0.8** are the fragments spanning from the initiating methionine of p2.45 to the *Pst*I site at 1462 and the *Stu*I site at 995, respectively.

pC2.24 resulted from an exoIII deletion series made from the C-terminal end of the coding sequence of p1.15 and contains the residues from 1459 to 2278.

The initiating methionine was deleted from the polylinker of pREP3 by digestion with *Bal*I and *Bam*HI, treatment with the Klenow fragment of DNA polymerase I, and self ligation. This recreates the *Bam*HI site and deletes the ATG which lies between the two restriction sites.

RESULTS

The sequence of p85^{cdc10} may be divided into three main domains. First, the N-terminus, which has been proposed to be responsible for DNA binding by virtue of sequence homology with *SWI4*p (35). Second, the central region of the protein, which contains the two complete copies and two partial copies of the *cdc10/SWI6* motif, to which 11 of 13 temperature sensitive mutants have been mapped (27), and third, the C-terminus, where the two other *cdc10* thermosensitive mutants map, which contains a putative leucine zipper (50) and, C-terminal to it, a second helical region.

The observation that 11 of 13 *cdc10*^{ts} mutants sequenced are located in conserved residues of the *cdc10/SWI6* motif, while the remainder affect the C-terminal region (27), prompted us to study their effects upon the activity of the *S.pombe* DSC-1 complex. To study the effect of inactivation of p85^{cdc10}, protein extracts from mutants representing each of the 8 alleles (27) were assayed for their ability to bandshift a probe from the *cdc22* promoter region, before and after incubation at 35°C. The results are shown in figure 1. First, it is apparent that all the *cdc10*^{ts} mutants are impaired in DSC-1 activity compared with wild type (figure 1, compare lane 1 with lanes 2−9); the *cdc10MBC4* mutant shows no detectable bandshift, while the levels in *cdc10V50* are less than 5% of wild type (figure 1, lanes 1, 3 and 9), suggesting that these complexes are highly labile *in vitro*. The DSC-1 activity is strongly reduced in *cdc10P10*, *cdc10M71* and *cdc10K28* (figure 1, lanes, 2, 4 and 8) and decreased in *cdc10-129*, *cdc10K30* and *cdc10M47* (figure 1, lanes 5, 6, 7). Incubation of the extracts at 35°C before performing the bandshift assay abolished the activity in extracts of *cdc10V50*, *cdc10P10*, *cdc10M71* and *cdc10K28* and greatly reduced it in extracts of *cdc10-129* and *cdc10M47*, while that prepared from wild type cells was unaffected (figure 1, lanes 1, 10−18). In contrast, preincubation of *cdc10K30* extracts lead to only a slight reduction in activity compared with that observed at 0°C, though the overall level of activity was reduced compared with wild type extracts at both temperatures (figure 1, compare lanes 1 and 10 with lanes 6 and 15). The two mutations which reduce the activity of the DSC-1 complex to the greatest extent at 0°C, *cdc10V50* and *cdc10MBC4*, are located in the first *cdc10/SWI6* motif, and the C-terminal domain respectively. The *cdc10MBC4* mutation truncates the C-terminal 61 amino acids (27) and disrupts the helical domain, but leaves the putative leucine zipper intact (27). These observations confirm and extend those made previously

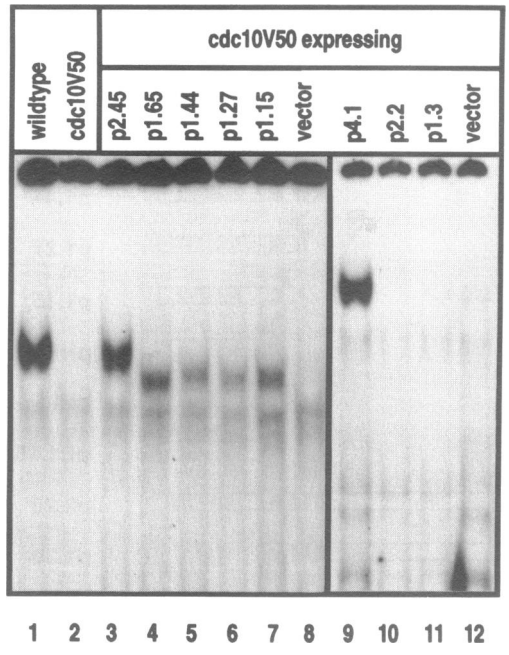

Figure 3. Rescue of the thermolabile bandshift in *cdc10*^{ts} alleles does not require the *cdc10/SWI6* repeats. Gel retardation assays were performed on extracts from *cdc10V50* cells transformed with pDW232 based plasmids expressing the indicated fragments. Note that plasmids encoding the *cdc10/SWI6* motif and C-terminal domains will restore DSC-1 activity and that complexes smaller than that seen with full length p85^{cdc10} are formed in these cases.

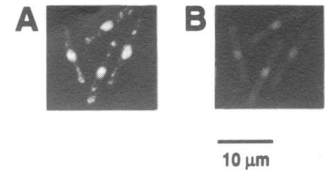

Figure 4. Mutant p85^{cdc10} is nuclear. Exponentially growing *cdc10MBC4* cells were shifted from 25°C to 36°C for 4 hours and fixed and processed for indirect immunofluorescence as described in materials and methods. Panel A, cells stained with DAPI; panel B, cells stained with anti p85^{cdc10}. The scale bar represents 10 μm. Identical staining was seen at 25°C in *cdc10MBC4* and at 25°C and 36°C in representatives of all the other seven alleles (not shown).

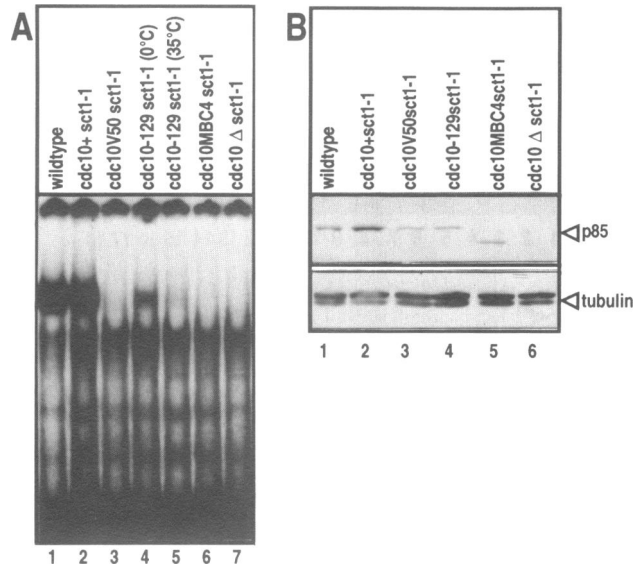

Figure 5. The *sct1* suppressor of p85^{cdc10} does not rescue thermolabile bandshift activity *in vitro*. Panel A. Gel retardation assays were performed upon extracts of the indicated strains. The *cdc10-129* protein extract was tested either before or after preincubation at 35°C for 5 minutes. The *sct1-1* mutation has no effect upon the DSC-1 bandshift activity. Preincubation at lower temperatures gave similar results (not shown). Panel B. Western blots of the extracts used for the experiment shown in panel A. The top panel was probed with antiserum to p85^{cdc10}, while the bottom one was probed for tubulin, as loading control. Note that the product of the *cdc10MBC4* gene is shorter than wild type, as predicted by the DNA sequence (27) and that the levels of the mutant p85^{cdc10} proteins are similar to those seen in a *sct1*$^{+}$ background.

on *cdc10-129*, *cdc10MBC4* and *cdc10K28* (40) and demonstrate that mutations in the *cdc10/SWI6* motifs, the leucine zipper or the C-terminal helical region all impair DSC-1 activity *in vitro*, implicating these regions as important for the normal function of the DSC-1 complex.

To define which domains of p85^{cdc10} are important for the formation of the DSC-1 complex, we investigated the ability of the fragments of the *cdc10* gene depicted in figure 2, which express one or more of the domains discussed above, to restore DSC-1 bandshift activity in the *cdc10*^{ts} mutants. Extracts from the *cdc10V50* mutant show very little bandshift activity even at the permissive temperature (figure 3, lane 2). Expression of full length p85^{cdc10} restored bandshift activity to the *cdc10V50* extracts, whereas expression of vector did not (figure 3, lanes 3, 9). However, C terminal truncations, either the N-terminal fragment lacking the helical domain (p1.3), or a truncation of the last 38 amino acids (p2.2) did not restore bandshift (figure 3, lanes 10, 11). Plasmids encoding only the C-terminal helical domain and all or part of the *cdc10/SWI6* motif domain restored bandshift (p1.65, p1.44, p1.27, p1.15; figure 3, lanes 4–7), though not to the same extent as expression of full length p85^{cdc10}. The bandshifts produced by expression of p1.65, p1.44, p1.27 and p1.15 are smaller than that produced by expression of full length p85^{cdc10}, consistent with the formation of a complex incorporating the shorter *cdc10* proteins. A similar result was obtained for pE1, which encodes only the C-terminal helical domain (data not shown). Rescue of bandshift activity by these plasmids in *cdc10MBC4* and *cdc10-129* backgrounds gave similar conclusions (not shown).

p85^{cdc10} is a nuclear protein (AR, J. Marks and VS, EMBO J., submitted). Therefore, we tested whether the mutant p85^{cdc10} proteins are correctly localised at the restrictive temperature. A typical result, for the *cdc10MBC4* allele at 36°C, is shown in figure 4. Examination of mutants representative of the 8 alleles (27) indicated that the protein was in all cases nuclear at both the permissive and restrictive temperatures, showing that the temperature sensitive phenotype of *cdc10* mutants does not result from mislocalisation of p85^{cdc10}. The sequence of p85^{cdc10} does not contain any of the classical nuclear targetting signals (18) and the region of the protein responsible for nuclear targetting is unknown. The observation that the truncated *cdc10MBC4* protein, lacking the C-terminal 61 amino acids (27), is nuclear suggests these amino acids are not necessary for transport of p85^{cdc10} to the nucleus.

Mutations in the *sct1* gene can bypass the requirement for

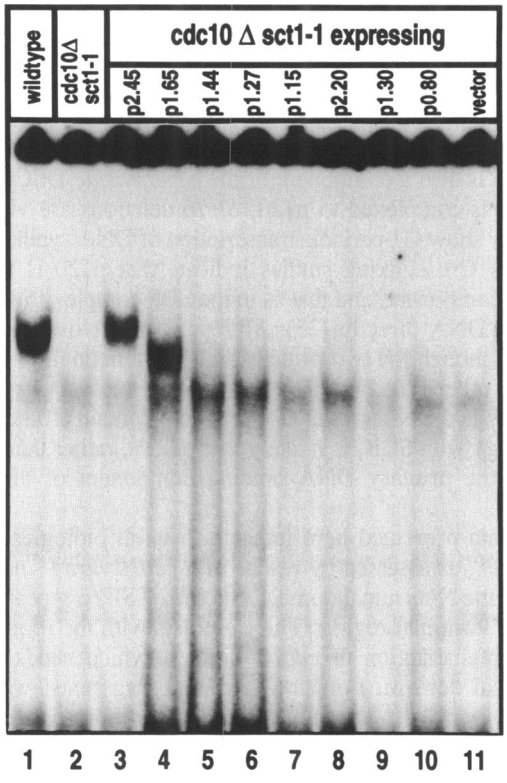

Figure 6. Rescue of bandshift activity in a *cdc10* null background requires both the *cdc10/SWI6* boxes and the C-terminal helical domain. Gel retardation assays were performed on extracts prepared from *cdc10::ura4*$^+$ *sct1-1* cells transformed with pREP3 based plasmids, expressing the indicated fragments of p85^{cdc10}. Note that the bandshift is only restored by expression of either full length p85^{cdc10} or the *cdc10/SWI6* and C-terminal region (lane 4).

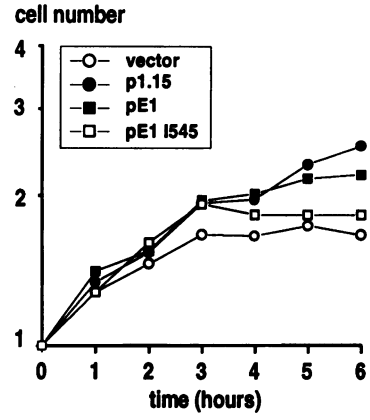

Figure 7. Rescue of *cdc10*^{ts} mutants by truncated fragments of p85^{cdc10}. Cells transformed with the indicated plasmids were grown to mid-exponential phase at 25°C and shifted to 36°C. Cell number was monitored using a Coulter counter. The cell number is indicated in arbitrary units; the cell number at the start of the experiment was 1.2×10^6 per ml. Note that cells transformed by p1.15 continue to divide after shift to 36°C, while cells transformed with the vector alone do not. Cells transformed with a plasmid expressing only the C-terminal domain (pE1) also continue to divide, though at a reduced rate compared with p1.15. Mutation of the initiating methionine of pE1 abolishes this complementation (pE1 I545).

p85^{cdc10} for cell cycle progression (11, 12). We therefore investigated the effect of *sct1-1* mutation upon the DSC-1 bandshift activity. In a *cdc10*$^+$ background, *sct1-1* did not interfere with DSC-1 activity (Figure 5A, lane 1, 2), and crossing the *sct1-1* mutation into *cdc10MBC4* or *cdc10V50* did not restore bandshift activity (Figure 5A, lanes 3 and 6). In a *cdc10-129* background, the bandshift was decreased compared with wildtype and thermolabile after incubation at 35°C (Figure 5A, lanes 4, 5). These results are similar to those obtained in a *sct1*$^+$ background (see figure 1, lanes 1, 3, 5, 9, and 14), indicating that the *sct1-1* allele does not function by restoring full activity to the DSC-1 complex. Western blotting showed that *sct1-1* does not act by increasing the levels of the mutant forms of p85^{cdc10} (figure 5B). Interestingly, the level of p85^{cdc10} is slightly increased in a *cdc10*$^+$ *sct1-1* background. Previous studies have demonstrated that cells will tolerate high levels of overexpression of p85^{cdc10} (more than 10 fold) with only a slight cell cycle delay in G1 while lower levels have no effect (19; VS and AR, unpublished data), so the biological significance of the small (<2 fold) increase observed is at present unclear.

No bandshift activity was detected in extracts of *cdc10::ura4*$^+$ *sct1-1* cells, where there is no p85^{cdc10} (figure 5A, lane 7). This indicates that the mutant *sct1-1* protein alone is incapable of forming a stable DSC-1 complex *in vitro*.

Since extracts of *cdc10::ura4*$^+$ *sct1-1* cells produce no DSC-1 bandshift (figure 6, lane 2), we investigated whether ectopic expression of p85^{cdc10}, or fragments of it, would rescue the bandshift activity. Expression of full length p85^{cdc10} restored wild type levels of bandshift activity (figure 6, lanes 1−3), showing that the observed bandshift is dependent upon the presence of p85^{cdc10}. Expression of p85^{cdc10} molecules lacking either the last 38 amino acids (p2.2) or the C-terminal helical domain (p0.8, p1.3) did not restore activity (figure 6, lanes 8, 9, 10). When plasmids expressing fragments of the *cdc10* gene lacking the N-terminal domain were expressed, only p1.65, which encodes the C-terminal domain and the complete *cdc10/SWI6* repeat domain, was capable of restoring a bandshift (figure 6, lane 4). As observed when this fragment of p85^{cdc10} was expressed in the *cdc10V50* background (figure 3, lane 4), the bandshift is smaller than that produced by expression of full length p85^{cdc10} (compare figure 6, lanes 3 and 4), consistent with formation of a bandshift complex incorporating the truncated protein. Truncation of the repeats abolished the bandshift (p1.44, p1.27, p1.15; figure 6, lanes 5, 6, 7), suggesting that they are required for p85^{cdc10} function in the DSC-1 complex. This contrasts with the observation made in the *cdc10*^{ts} background, where the repeats were shown to be dispensible for the function of the DSC-1 complex (figure 3, lanes 5−7). In addition, despite the fact that the protein encoded by the p1.65 fragment is larger than the others, the bandshift complex formed by expression of p1.65 in a *cdc10V50* background is smaller than those formed by expression of p1.44, 1.27 or p1.15 (compare figure 3, lane 4, with lanes 5−7). These observations could be explained if the p1.65 encoded protein is capable of forming a DSC-1 like complex in the absence of full length p85^{cdc10} molecules, while the proteins encoded by p1.44, p1.27 and p1.15 require the presence of p85^{cdc10}, even if it is mutant. Taken together, these data are consistent with the view that the *S.pombe* DSC-1 complex contains at least two molecules of p85^{cdc10}.

The biological activity of the fragments of p85^{cdc10} depicted in figure 2 was assessed by testing their ability to rescue *cdc10* temperature sensitive and null mutants. The results are shown in table 1. Deletions which truncated the N-terminal domain,

Table 1.

	COMPLEMENTATION	
PLASMID	cdc10ts	cdc10delta
p4.1	+	+
p2.45	+	+
p1.65	+	-
p1.44	+	ND
p1.27	+	ND
p1.15	+	-
pN19	+	ND
pE1	+	ND
pE1 I545	-	ND
p2.2	+	+
p1.3	-	-
p0.8	-	ND
pC2.24	-	ND

Cells were transformed with the plasmids expressing the indicated fragments of p85^{cdc10} from DW232 and allowed to form colonies at 25°C. For complementation of *cdc10^{ts}* mutants, cells were shifted to the restrictive temperature, 36°C, and to assess rescue of the null allele, cells were induced to undergo meiosis and spores plated out. A (+) indicates that a clone could rescue the mutation, (−), that it could not. ND indicates that a clone was not tested. For complementation of *cdc10^{ts}* mutants, *cdc10-129*, *cdc10M47*, *cdc10K28* and *cdc10MBC4* were tested, with identical results for all four strains.

leaving only the *cdc10/SWI6* and C-terminal helical region, were able to rescue all *cdc10^{ts}* mutants (consistent with previous observations on the *cdc10-129* allele; 18). Further deletions which removed the *cdc10/SWI6* repeats, leaving only the C-terminal domain, were still able to complement, though with reduced efficiency (pE1, pN19; table 1; figures 2 and 7). Clones which expressed only the N-terminal domain of p85^{cdc10} (p0.8) or the N-terminal domain and the *cdc10/SWI6* repeats (p1.3), were unable to complement any of the *cdc10^{ts}* mutants tested (table 1). Biological function was retained following small deletions at the C-terminus of p85^{cdc10} in the full length protein (p2.2) but not in a clone expressing a truncated form of the protein (pC2.24). These data are in broad agreement with the ability of the fragments to rescue the bandshift activity in a *cdc10^{ts}* background (figure 3). The exception to this, p2.2, is discussed below. When the much more stringent criterion of rescue of the null allele was imposed, it was found that only full length p85^{cdc10} (p2.45) and p2.2, which deletes 38 amino acids at the C-terminus, were still functional; all other clones were incapable of rescuing a *cdc10::ura4^{+}* null allele (table 1).

DISCUSSION

p85^{cdc10} is a component of the *S.pombe* DSC-1 complex, which is thought to mediate periodic transcription of certain genes in late G1. In order to understand the role of p85^{cdc10} in the function of this complex, we have analysed which domains of p85^{cdc10} are required for biological activity and the formation of a stable DSC-1 complex *in vitro*, in *cdc10* thermosensitive and null backgrounds.

A model for the understanding of the DSC-1 complex is provided by studies of the *S.cerevisiae* SBF complex (SWI4/SWI6 binding factor). The latter is a complex of the *SWI4*p and *SWI6*p proteins, and is responsible for the transcription of the *HO* endonuclease and *CLN* genes in G1. Analysis of the roles of different domains of *SWI4*p and *SWI6*p in the SBF complex has shown that the N-terminal domain of *SWI4*p, from amino acids 36 to 168, is the primary DNA recognition component; *SWI6*p does not bind directly to DNA. The interaction of *SWI4*p and *SWI6*p is mediated through the interaction of their C-terminal domains. Integrity of the *cdc10/SWI6* motifs is required for formation of a SBF complex which is capable of binding DNA, but not for the interaction of *SWI4*p and *SWI6*p (50−52).

*SWI6*p is also a component of the *S.cerevisiae* DSC-1 factor, where it is complexed to p120; *SWI6* deletions are viable, but no longer show G1 periodic transcription of DNA synthesis genes (34, 35). Crosslinking studies indicate that p120 is the DNA binding component, and that as in the SBF complex, *SWI6*p does not bind DNA directly (35). *SWI6*p is most closely related to p85^{cdc10}, though the two proteins will not functionally substitute for each other (20), and p72^{sct1} is most homologous to *SWI4*p, especially in the N-terminal DNA binding domain (10, 11). Thus, by analogy with SCB, it is likely that p72^{sct1}, rather than p85^{cdc10} will be the primary DNA binding component of the DSC-1 complex.

The data presented here indicate that full biological activity of p85^{cdc10}, as assessed by rescue of a *cdc10::ura4^{+}* null allele, requires the N-terminal domain, the *cdc10/SWI6* repeats and the helical C-terminal region. This contrasts with the requirements for complementation of *cdc10^{ts}* alleles, which needs only the C-terminal domain. Longer C-terminal fragments, which also encode part of the *cdc10/SWI6* domain give better complementation of *cdc10^{ts}* mutants, suggesting that the repeats are also important for p85^{cdc10} function in the DSC-1 complex. The N-terminal domain, either alone or in combination with the *cdc10/SWI6* motifs, does not have biological activity.

Mutations in either the C-terminal region or the *cdc10/SWI6* motifs of p85^{cdc10} cause conditional defects in cell cycle progression. All of the *cdc10^{ts}* mutations we have assayed affect the activity of the DSC-1 complex *in vitro*, suggesting that the cell cycle arrest may result from lack of DSC-1 function at the non permissive temperature.

The ability of fragments of p85^{cdc10} to restore the DSC-1 bandshift in a *cdc10^{ts}* background correlates well with the biological assays. Fragments encoding the C-terminal domain alone are sufficient to restore a bandshift, while those which lack this domain are unable to do so. These data suggest that the C-terminal domain is important both for biological function and formation of the DSC-1 complex. The protein encoded by p2.2, which has a deletion of 38 amino acids at the C-terminus, is able to rescue all *cdc10^{ts}* mutants, but does not restore the DSC-1 bandshift activity *in vitro*. This result is similar to that obtained from the *cdc10MBC4* mutant, where truncation of the C-terminal 61 amino acids abolishes the bandshift *in vitro*, and suggests that these small deletions destabilise the DSC-1 complex *in vitro*. The p2.2 protein and the *cdc10MBC4* mutant proteins may be functional *in vivo* due to the presence of the *cdc10/SWI6* repeat domain; a truncated version of the p2.2 protein lacking the complete *cdc10/SWI6* domain and N-terminus (pC2.24) is unable to provide biological function, or restore DSC-1 bandshift. This suggests that the *cdc10/SWI6* domain also contributes to the stability of the DSC-1 complex.

Mutations in the *sct1* gene can bypass the requirement for p85^{cdc10} for cell cycle progression (11, 12). The *sct1-1* mutation (12), did not restore bandshift activity to those mutants in which the complex is defective (*cdc10MBC4*, *cdc10V50*), or rescue the thermolabile DSC-1 activity in a *cdc10-129* mutant, indicating that it does not act by stabilizing the mutant DSC-1 complex. This differs from the effect of the *sct1-b1* mutation, which will

restore partial activity *in vitro* to extracts of *cdc10-129* cells (11). The *sct1-1* mutation has not been sequenced, so it is possible that this discrepancy results from differences in the nature of the *sct1* mutants used in the two studies. No DSC-1 bandshift activity was found in *cdc10::ura4*$^+$ *sct1-1* cells (which lack p85^{cdc10}), indicating that the mutant protein encoded by the *sct1-1* allele is not capable of forming a stable DSC-1 complex. As in the *cdc10*^{ts} background, fragments of p85^{cdc10} lacking the C-terminal domain were unable to rescue the DSC-1 bandshift activity. However, in contrast to the result obtained in a *cdc10* temperature sensitive background, we found that the C-terminal domain alone was incapable of restoring a DSC-1 bandshift; only expression of the C-terminus and both complete copies of the *cdc10/SWI6* repeats gave a bandshift, forming a complex that migrated more rapidly than that resulting from expression of full length p85^{cdc10}. These data suggest that in the presence of the *sct1-1* mutation, the N-terminal domain of p85^{cdc10} is not essential for the bandshift activity and that in the absence of a full length p85^{cdc10}, formation of a bandshift complex requires the integrity of both the *cdc10/SWI6* motifs and the C-terminal domain, and further support the view that the *cdc10/SWI6* domain contributes to the formation of a DSC-1 complex that is stable *in vitro*.

Expression of some truncated forms of p85^{cdc10} in *cdc10*^{ts} mutants leads to the formation of a DSC-1 complex which is smaller than that formed by expression of full length p85^{cdc10}. These complexes are not formed when the fragments are expressed in the *cdc10::ura4*$^+$ *sct1-1* background, where p85^{cdc10} is absent, suggesting that in the *cdc10*^{ts} background, they are formed by the interaction of the mutant p85^{cdc10} protein and the expressed fragment. This leads us to propose that there is more than one molecule of p85^{cdc10} in the DSC-1 complex.

By analogy with the SBF complex, we propose that the C-terminal domain of p85^{cdc10} mediates the protein−protein interactions required to form the DSC-1 complex, either with p72^{sct1} or another molecule of p85^{cdc10}. Our data also imply that the *cdc10/SWI6* motifs play an inportant role in the formation of the DSC-1 complex; mutations in this domain impair the function of the DSC-1 complex, both *in vivo* and *in vitro*, and this domain is essential for the formation of a DSC-1 like complex in the absence of p85^{cdc10}. The *cdc10/SWI6* motif mediates protein−protein interactions in other systems (28−31), so it is possible that in the DSC-1 complex their role is either to provide additional contacts with p72^{sct1}, or between molecules of p85^{cdc10}, which stabilise the complex.

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