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Domains Determining the Functional Distinction of the Fission Yeast Cell Cycle "Start" Molecules Res1 and Res2

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In *Schizosaccharomyces pombe* the "start" of the cell cycle is regulated by two parallel, functionally overlapping complexes composed of Res1-Cdc10 and Res2-Cdc10. Res1 and Res2 are structurally very homologous and are required for the start of the mitotic and meiotic cycle, respectively. We have addressed the question which parts of the proteins are essential for function and determine the functional specificity. Several discrete domains in the nonconserved C-terminal region are essential for the mitotic and meiotic start function and determine the functional specificity independently of the structurally conserved motifs at the N-terminal end and in the center. One of these domains in Res2 restricts Res2 to interact only with Rep2. Res2 without this domain behaves like a functional chimera having the properties of Res2 and Res1. Likewise, internally truncated forms of Res1 lacking the centrally located ankyrin repeats and adjacent sequences can partially suppress the meiotic defect in $res2^-$ cells. These truncated Res1 molecules behave like functional chimeras with the properties of Res1 and Res2.

INTRODUCTION

In the G_1 phase of the cell cycle, a cell determines which pathway it will take; it will either start a new round of cell proliferation or choose alternative pathways, leading to cell arrest and differentiation. The decision which pathway to take is made at a point in G_1 called "start." Once this point is passed a cell is committed to the mitotic cycle. It proceeds from the G_1 to the S phase and a new round of cell propagation will occur. No alternative routes can be taken until the cell returns to G_1. In the fission yeast *Schizosaccharomyces pombe*, several genes are involved in the execution of start. One of these is the $cdc2^+$ gene, encoding a cyclin-dependent protein kinase which is essential for the progression from G_1 to S phase and from G_2 to M phase (Nurse *et al.*, 1976; Nurse and Bissett, 1981; Nurse, 1990). The others are the $cdc10^+$, $res1^+/sct1^+$, and $res2^+/pct1^+$ genes (Aves *et al.*, 1985; Tanaka *et al.*, 1992; Caliguri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). Temperature-sensitive mutants of the $cdc10^+$ gene arrest at start in the G_1 phase of the cell cycle upon incubation at their restrictive temperature, and cells deleted of this gene are nonviable (Marks *et al.*, 1992; Tanaka *et al.*, 1992). The *S. pombe* start genes $res1^+$ and $res2^+$ encode proteins of similar size with overlapping, though distinct, functions. The two molecules are structurally homologous to each other as well as to Cdc10 and the *Saccharomyces cerevisiae* proteins SWI4, SWI6, and Mbp1 (Andrews and Herskowitz, 1989; Dirick *et al.*, 1992; Koch *et al.*, 1993). They all share the ankyrin repeats, two centrally located highly conserved motifs of 33 amino acids. In addition, Res1, Res2, SWI4, and Mbp1 contain a highly conserved N-terminal domain of 62 amino acids that is involved in the binding of these molecules to DNA. Heterodimeric Res1-Cdc10 and Res2-Cdc10 as well as Mbp1-SWI6 complexes (Lowndes *et al.*, 1992a,b; Verma *et al.*, 1992; Caliguri and Beach, 1993; Koch *et al.*, 1993; Reymond *et al.*, (1993); Zhu *et al.*, 1994; Ayte *et al.*, 1995) interact with the MCB box, a *cis*-acting transcriptional element in the promoters of

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several cell cycle-dependent genes which are required for the progression from G_1 to S-phase (Gordon and Fantes, 1986; Lowndes *et al.*, 1991; McIntosh *et al.*, 1991; for review, see Andrews, 1992; Kelly *et al.*, 1993; Hofmann and Beach, 1994).

Res1 and Res2 are structurally very homologous in their N-terminal region and the centrally located ankyrin repeats, but they differ in their C-terminal domain. Although they are functionally overlapping with respect to their mitotic role, the Res2 molecule functions mainly during meiosis. For the Res1 molecule, on the other hand, no meiotic role has been established. Thus, fission yeast contains two structurally related and functionally overlapping start systems represented by the Res1-Cdc10 and Res2-Cdc10 complexes which are crucial for the mitotic and meiotic cycles, respectively.

Cells carrying a disrupted *res1*$^+$ allele arrest at start in the mitotic cycle and show severe growth defects at 21 and 36°C (Tanaka *et al.*, 1992). *res2*$^-$ cells are viable, but they are partially defective in the start of premeiotic DNA synthesis and a later step in meiosis (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). They are unable to proceed through the normal meiotic steps and will form asci with less than four spores. However, the double disruptant of both genes is nonviable due to the shared redundant mitotic function of both molecules (Miyamoto *et al.*, 1994).

In *S. pombe*, the Rep2 protein functions as a transactivator that interacts predominantly with the Res2-Cdc10 complex (Nakashima *et al.*, 1995). It binds to the Res2 protein in vitro and forms complexes with Res2-Cdc10 and Res1-Cdc10 in vivo. Rep2 is the major transactivator subunit for the Res2-Cdc10 complex in the mitotic cycle. Consequently, without Rep2, Res2-Cdc10 has no cell cycle start function.

To interpret the events which take place when cells proceed through start and commit themselves to a new round of cell propagation, it is important to understand the roles of the Res1 and Res2 molecules, their functional specificity, regulation, and interactions with other components of the cell cycle machinery. One way to approach this question is to identify domains essential for function and determine which molecular structures of the two proteins define functional specificity and how are these related to the overall composition of the molecules. Our approach was to generate various derivatives of the *res1*$^+$ and *res2*$^+$ genes by truncation and to analyze these constructs by overexpression from the SV40 promoter in different *S. pombe* backgrounds with either a mitotic or meiotic defect. This approach allowed us to determine which parts of the two proteins contain domains essential for their function. Chimeric molecules of Res1 and Res2 and their overexpression in different mitotic and meiotic defective *S. pombe* backgrounds made it possible to identify the domains that determine the functional specificity. We report here the localization of these domains, their functional specificity, and interaction with the structurally conserved domains at the N-terminal end and in the center of the two proteins.

MATERIALS AND METHODS

Strains, Media, and Vectors

The *S. pombe* strains used in this study are listed in Table 1. Media were prepared as described (Egel and Egel-Mitani, 1974; Gutz *et al.*, 1974; Moreno *et al.*, 1990; Okazaki *et al.*, 1990). Media containing 1.5% agar were used for plating. *Escherichia coli* was grown in LB (0.5% yeast extract, 0.5% NaCl, 1% tryptone); for plates, 1.5% agar was added. The pcL vector containing the SV40 promoter has been described (Okazaki *et al.*, 1990; Igarashi *et al.*, 1991; Nagata *et al.*, 1991). The pAlter-1 and pBluescript vectors were purchased from Promega (Madison, WI) and Stratagene (Cambridge, United Kingdom), respectively.

DNA Manipulation

All DNA manipulations were done using standard molecular biology techniques (Sambrook *et al.*, 1989). For overexpression in *S. pombe*, all constructs were expressed from the SV40 promoter. For the construction of the Res1-Res2 and Res2-Res1 chimeric constructs the following general approach was chosen: Using mutagenic oligos and the pAlter-1 in vitro mutagnesis system from Promega, *Eco* 105I restriction sites were introduced into *res1*$^+$ and *res2*$^+$ at the indicated positions. The genes with the newly introduced *Eco* 105I restriction site were subcloned from the pAlter to the pcL vector. The chimeric constructs were generated by digestion with *Eco* 105I and *Xho*I and subsequent swapping of the respective domains of Res1 and Res2. The *Xho*I site is a cloning site in the pcL vector and is not present in either *res1*$^+$ or *res2*$^+$. In each case, the DNA of the swapped domain contained at the 5′ end the *Xho*I and at the 3′ end the *Eco* 105I site. The internally truncated *res1*$^+$ and *res2*$^+$ genes were generated in a similar way by the introduction of two *Eco* 105I restriction sites at the indicated positions in pAlter-*res1*$^+$ and pAlter-*res2*$^+$, subcloning into pcL, and digestion with *Eco* 105I. The constructs with the respective fragments deleted were religated. Res1 and Res2 molecules which were truncated at the C-terminal end were generated by introduction of a stop codon followed by a *Bam*HI restriction site at the indicated positions. The construct was digested either with *Bam*HI (in the case of *res1*$^+$) or *Bam*HI and *Xho*I (in the case of *res2*$^+$) and the truncated genes were subcloned from pAlter to the pcL vector. The conservation of the correct reading frame was confirmed by sequencing the final constructs using the Sequenase DNA sequencing system from Amersham (Amersham, United Kingdom).

Suppression of res1$^-$ *and rep2*$^-$ *Cells by res1*$^+$, *res2*$^+$, *or Various Derivatives*

The ability to suppress the cold sensitive growth defects of *res1*$^-$ and *rep2*$^-$ cells was assayed as follows: The K123-14D and N3-141S

Table 1. Strains used in this study

Strain	Genotype
K123-14D	h^{-S} *ade6*-M126 *ura4*-D18 *leu*-1-32 *res1*::*ura4*$^+$
K156-D1	h^{-S} *leu1*-32 *ura4*-D18 *res1*::*ura4*$^+$
N3-141S	h^{-S} *leu1*-32 *ura4*-D18 *rep2*::*ura4*$^+$
N5-211C	h^{+S} *leu1*-32 *ura4*-D18 *rep2*::*ura4*$^+$
M105	h^{-S}/h^{+N} *ade6*-M210/*ade6*-M216 *ura4*-D18/*ura4*-D18 *leu1*-32/*leu1*-32 *res1*$^+$/*res1*::*ura4*$^+$ *res2*::*ura4*$^+$/*res2*$^+$
M106	h^{90} *ura4*-D18 *res2*::*ura4*$^+$ leu1-32

strains were grown overnight to mid-log phase in Edinburgh minimal medium (EMM) (Moreno *et al.*, 1990) containing 50 mg/l of adenine, histidine, leucine, lysine, and uracil and transformed with the indicated constructs using lithium acetate (Okazaki *et al.*, 1990). All cells were incubated on selective minimal medium plates overnight at 30°C. After 16 h (for *res1*$^-$ cells) or 18 h (for *rep2*$^-$ cells), one-half of the plates was shifted to the restrictive temperature of 21°C (for *res1*$^-$) or 18°C (for *rep2*$^-$). After several days the numbers of Leu$^+$ colonies at the permissive and restrictive temperature were counted and the percentage ratio was determined. Experiments were repeated several times and the values represent the means ± SD of several independent experiments.

Suppression of the Meiotic Defect in h^{90}res2$^-$ Cells

S. pombe strain M106 was transformed with the indicated constructs and Leu$^+$ colonies selected at 30°C on minimal medium plates. To induce conjugation and subsequent sporulation, Leu$^+$ colonies were spotted onto SSA plates (Egel and Egel-Mitani, 1974; Gutz *et al.*, 1974) and incubated at 30°C. After 1–2 days, the cells were analyzed microscopically and the number of asci containing four spores or less than four spores was determined. For each construct tested, at least 300 asci were counted and the experiments were repeated several times. The values represent the means ± SD of several independent experiments.

Suppression of Double Mutants

S. pombe res1$^-$ (K156-D1) and *rep2*$^-$ (N5-211C) cells were transformed with the indicated constructs and Leu$^+$ colonies were selected at 30°C on minimal medium plates. The transformed cells were subsequently spotted onto SSA plates to induce conjugation and sporulation and incubated for several days at 30°C. After asci had formed, random sporulation was carried out and *res1*$^-$*rep2*$^-$ haploid segregants were germinated at 30°C. In a first screen, colonies were tested for the presence or absence of wild-type *res1*$^+$ or *rep2*$^+$ by polymerase chain reaction (PCR). Colonies with a genomic *res1*$^+$ gene were identified using a sense primer starting in the intron in the *Kpn*I-*Sac*I fragment of *res1*$^+$ (5′-TTTATCAATTTATACTTTTTTGGATGC-3′) and the antisense primer (5′-TTAAGATCCACTTTGATCTGTATT-3′) starting at the stop codon. *rep2*$^+$ candidates were identified by amplification of the complete open reading frame (ORF) of the gene with the sense primer (5′-ATGCATTTTGCAGACATTCCT-3′) and the antisense primer (5′-TTAAAAGAGCCAGTCATCTAT-3′) starting at the beginning or end of the ORF, respectively. The presence of the disrupted gene was confirmed by genomic Southern blot analysis. Genomic Southern blot analysis of the disrupted *res1*$^+$ gene was done as described previously (Tanaka *et al.*, 1992); to confirm the disrupted *rep2*$^+$ gene, genomic DNA was digested with *Sca*I and *Eco*RI. As a probe, the 535-bp *Afl*II-*Nhe*I fragment was used. *res1*$^-$*res2*$^-$ double disruptants were generated in a similar manner by transforming the diploid strain M105 with the respective constructs. *res2*$^+$ candidates were identified in a first screen by PCR using the sense primer (5′-CCATGGATTGTGTAAACTCT-3′) which starts at position 1621 of the ORF (Miyamoto *et al.*, 1994) and the antisense primer (5′-TTTCTCGGGTTAATGCTT-3′) which starts before the stop codon. The presence of the disrupted *res2*$^+$ gene was confirmed by genomic Southern blot analysis; genomic DNA was digested with *Eco*RI. As a probe, the 1.4-kb *Hin*dIII-*Eco*RI fragment was used (Miyamoto *et al.*, 1994). Neither the *res1*$^+$ and *rep2*$^+$ nor the *res1*$^+$ and *res2*$^+$ are linked. In case the double disruptant harboring the respective plasmid could be isolated, 50 colonies were tested by PCR for the absence of the wild-type gene, whereas in case the double disruptant strain could not be isolated at least 150 and up to 750 colonies were tested in an initial screen. *res1*$^-$*rep2*$^-$ and *res1*$^-$*res2*$^-$ double-disruptant strains harboring the respective construct were initially grown at 30°C and after verification of the disruption by genomic Southern blot streaked onto minimal medium plates and incubated at 18 or 21°C, respectively. Double disruptants grew at 30°C as well as 21°C or 18°C.

In Vitro Binding of GST-Rep2 to Res2 and Different Res2 Derivatives

Induction and purification of glutathione S-transferase (GST)-Rep2 for in vitro binding experiments were performed according to Nakashima *et al.* (1995). For in vitro transcription/translation, the indicated *res2*$^+$ derivatives were subcloned into pBluescript KS$^-$ as *Xho*I-*Bam*HI fragments to be transcribed from the T3 promoter. The respective proteins were synthesized by coupled transcription/translation (TNT System; Promega) and labeled with [^{35}S]methionine as recommended by the manufacturer. GST-Rep2 fusion protein bound to Res1, Res2, or the indicated Res2 derivatives was analyzed by 12.5% SDS-PAGE and autoradiography. The amount of bound material was determined visually and compared with the amount bound by full-length Res2. Arbitrarily, the amount of GST-Rep2 bound by Res2 was defined to be +++. In vitro-labeled Res2 proteins which bound less GST-Rep2 than full-length Res2 were defined as ++, +, or −.

RESULTS

C-Terminal Region of Res2 Contains Domains Essential for the Mitotic and Meiotic Function

To identify domains in the Res1 and Res2 proteins that are essential for function, we constructed various truncated forms of the two molecules and analyzed their biological activity in vivo by overexpression in different *S. pombe* host strains.

We first constructed mutants of Res2 progressively truncated at the C-terminus and assayed their mitotic function as measured by suppression of the cold-sensitive growth defect in *res1*$^-$ and *res1*$^-$ *res2* $^-$ double-disruptant cells. *res1*$^-$ and *res1*$^-$*res2*$^-$ cells allow detection of the Res1 or Res2 mitotic cycle start function of the molecule. The mitotic cycle start function of Res2, as assayed with the *res1*$^-$ host, remained intact until 41 amino acids were deleted from the C-terminus (Table 2). When more amino acids were deleted, truncated Res2 began to lose the ability to rescue *res1*$^-$ cells, and removal of 159 amino acids or more completely inactivated its start function.

Consistent results were obtained with the *res1*$^-$ *res2*$^-$ double-disruptant host (Table 2). These results show that a region upstream of the C-terminal 41 amino acids is essential for the function of Res2 in the mitotic cycle.

To identify the upstream boundary of the C-terminal region required for the mitotic function, internal deletion mutants of *res2*$^+$ were constructed and tested for their ability to rescue the *res1*$^-$ host. Internal deletions of various lengths were made from amino acid 400 (Figure 1) toward the C-terminus. Deletion of 40 amino acids did not affect the biological activity (Table 3). Further deletions, however, lowered the ability to rescue *res1*$^-$ cells, and deletion of 145 amino acids or more inactivated the protein.

The Res2 molecule is required for premeiotic DNA synthesis and subsequent meiosis and spore formation (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). Cells with a disrupted *res2*$^+$ gene will undergo abnormal meio-

Table 2. Deletion analysis of C-terminal functional domains in Res2

Construct	% Asci with 4 spores	% Suppression of *rep2*$^-$	% Suppression of *res1*$^-$	Suppression of *res1*$^-$ *res2*$^-$	Suppression of *res1*$^-$ *rep2*$^-$
pcLx empty vector	5 ± 2	<1	<1	no	no
pcLres1	2 ± 1	86 ± 1	92 ± 9	yes	yes
pcLres2	65 ± 3	<1	75 ± 7	yes	no
−12AA	40 ± 1	10 ± 5	94 ± 2	—	yes
−32AA	39 ± 1	31 ± 8	94 ± 3	yes	—
−41AA	32 ± 3	42 ± 4	90 ± 9	—	—
−76AA	14 ± 4	33 ± 12	49 ± 10	yes	yes
−116AA	14	14 ± 3	6 ± 3	—	no
−159AA	8 ± 2	1 ± 1	1 ± 1	no	—
−259AA	5 ± 3	2 ± 1	3 ± 3	—	—
−343AA	4 ± 3	<1	2 ± 2	no	—

The dark and light horizontal lines represent Res2 and Res1, respectively. The ankyrin motifs are represented by vertical bars in the center of the molecule. The number of amino acids in front of each constructs indicates the number of amino acids deleted from the C-terminal end. Yes and no indicates that the construct can suppress the double disruptant at 21°C or 18°C or that the double-disruptant strain could not be isolated, respectively. —, construct has not been tested.

sis, yielding asci with less than four spores. The meiotic defect in *res2*$^-$ cells can only be rescued by *res2*$^+$, but not by *res1*$^+$ (Miyamoto *et al.*, 1994). This assay will allow detection of the Res2-specific function of the protein.

The C-terminal part of Res2 was found to be essential for the meiotic function of the protein. The same C-terminal and internal deletion mutants of *res2*$^+$ (Tables 2 and 3) were tested for their ability to rescue the meiotic defect of *res2*$^-$ cells. Deletion of up to 41 amino acids from the C-terminal end significantly compromised the meiotic activity of the molecule. Thirty-two percent of the zygotes generated asci with four spores, whereas overexpression of untruncated *res2*$^+$ cDNA produces 65%. Further deletions severely affected this activity and removal of the entire C-terminal portion completely destroyed the meiotic function of Res2. These results show that at least the C-terminal 76 amino acids are required for the meiotic function.

Internal deletion of 40 amino acids starting at amino acid 400 toward the C-terminal end had no effect on the meiotic function of Res2, but additional deletions gradually reduced this activity (Table 3). A region spanning from amino acid 441 up to the C-terminus is required for the meiotic function of Res2.

C-Terminal Part of Res1 Determines the Mitotic Activity

We constructed C-terminal truncated and internally deleted Res1 molecules and assayed these for their mitotic ability as measured by suppression of the cold-sensitive growth defect in *res1*$^-$, *rep2*$^-$, and *res1*$^-$ *rep2*$^-$ double-disruptant cells (Table 4). Because the *res1*$^+$, but not the *res2*$^+$, gene can suppress the *res1*$^-$ and *res1*$^-$*rep2*$^-$ host, this assay will allow detection of the Res1-specific function of the protein. Truncation at the C-terminus progressively reduced the ability of Res1 to rescue *rep2*$^-$ and *res1*$^-$ cells. A deletion of 14 amino acids significantly lowered the ability to rescue *rep2*$^-$ cells without affecting the ability to suppress the cold-sensitive phenotype of the *res1*$^-$ host. Deletion of 33 (our unpublished data) and 45 amino acids resulted in an 8- to 9-fold reduction in the ability to rescue *rep2*$^-$ cells, with only a slight affect on the suppression of the *res1*$^-$ host.

Deletion of 162 amino acids further reduced the ability of Res1 to rescue *rep2*$^-$ and *res1*$^-$cells, but additional deletions up to amino acid 387 largely restored the ability to suppress the latter host (54%). Deletions beyond the second ankyrin motif completely inactivated the molecule.

Internal deletions were made from amino acid 387 toward the C-terminus (Figure 1 and Table 4). Deletion of 90 amino acids between amino acids 387 and 478 did not affect the ability of Res1 to rescue *rep2*$^-$and *res1*$^-$ cells, but additional deletions progressively reduced the ability to rescue *rep2*$^-$ cells. The ability to suppress the *res1*$^-$ host remained unchanged.

Because *res1*$^-$ *rep2*$^-$ double-disruptant cells are inviable (Nakashima *et al.*, 1995) and can only be rescued by overexpression of *res1*$^+$, we tested whether C-terminal truncated Res1 proteins could rescue the

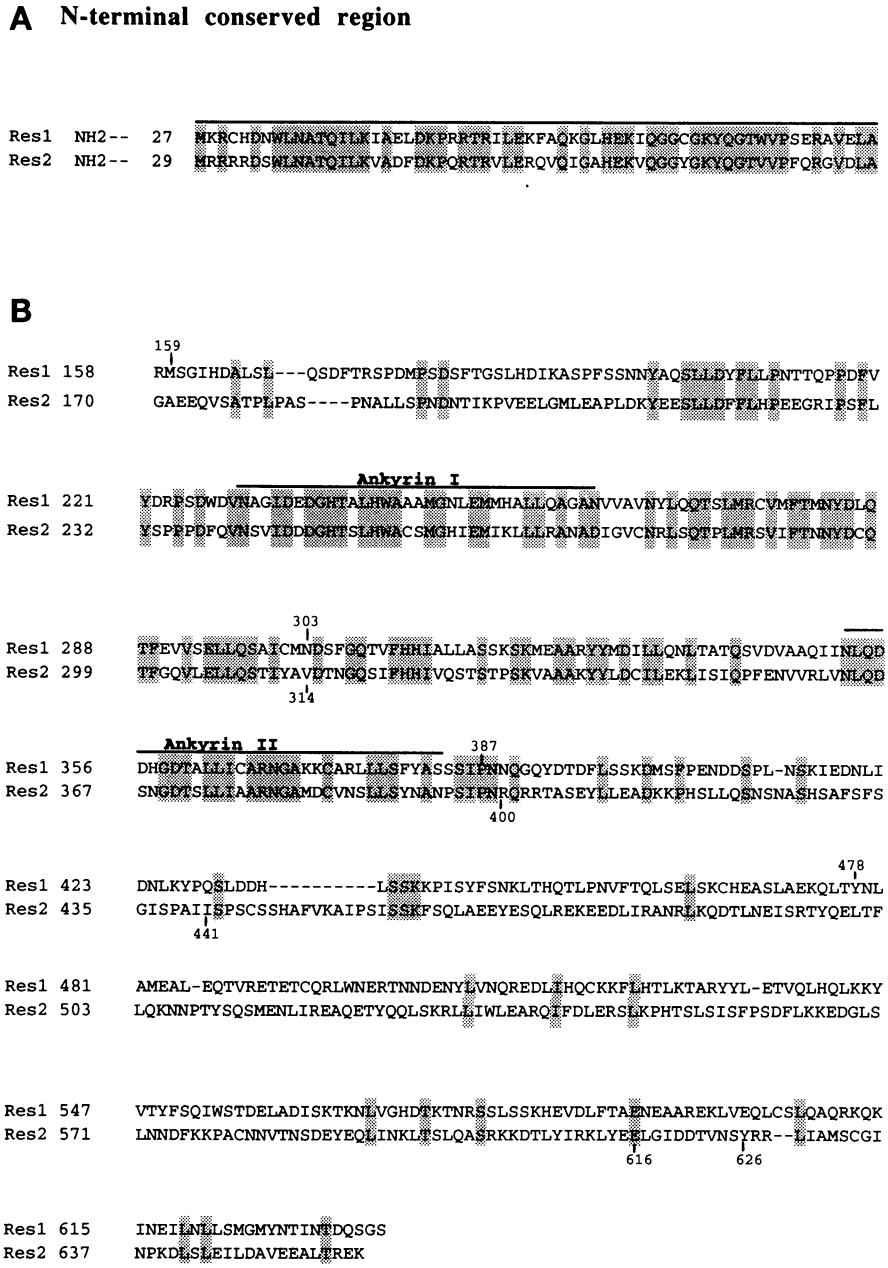


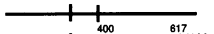
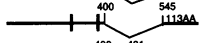
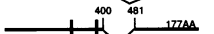
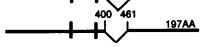
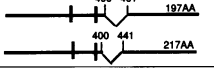
Figure 1. Structural comparison between Res1 and Res2. Alignment of the amino acid sequence of the N-terminal conserved (A) and the C-terminal region including the two ankyrin repeats (B). Amino acids shared between Res1 and Res2 are shaded. The N-terminal conserved region and the two ankyrin motifs are indicated by a horizontal line. The numbers represent the positions of amino acids described in Figure 3.

res1$^-$*rep2*$^-$ host at 18°C. Res1 proteins lacking the C-terminal 162 amino acids were still functioning as mitotic start molecules and could suppress the *res1*$^-$*rep2*$^-$ double disruptant. Truncated molecules with 250 amino acids deleted were unable to do so. However, these proteins could still function in cells with an intact *rep2*$^+$ gene and suppress the *res1*$^-$ host significantly. Res1 has a domain critical for its Rep2-independent mitotic activity, which resides between amino acid 478 and the C-terminus and is required for its cell cycle start function.

Functional Specificity of Res2 Is Determined by the C-terminal Part of the Molecule and Acts Independently of the DNA-binding Domain and the Ankyrin Motif

Res2 contains in the C-terminal region a domain that is essential for its meiotic function (Tables 2 and 3). This domain might confer the unique meiotic function to Res2. To examine this possibility, we constructed various chimeric molecules between Res1 and Res2 and tested these for their ability to function as a meiotic or

Table 3 Internal deletion analysis of Res2

Construct	% Asci with 4 spores	% Suppression of res1⁻ cells
pcLX empty vector	6 ± 1	<1
pcLres2	64 ± 1	75 ± 11
	19 ± 8	<1
	24 ± 1	8 ± 2
	24 ± 3	20 ± 3
	42 ± 6	22 ± 10
	64 ± 2	80 ± 6

An interrupted dark line represents internally truncated Res2. The numbers above the bars indicate the amino acids at the beginning and the end of the deletion and numbers above the horizontal line correspond to the number of amino acids in the depicted fragment.

mitotic suppressor (Table 5). The Res1-Res2 chimera made by splicing in the middle between the two ankyrin motifs behaved like Res2 and suppressed the meiotic defect of *res2*⁻ cells, but not the cold-sensitive phenotype of *rep2*⁻ cells. In contrast, the Res2-Res1 chimera made by the exact opposite combination behaved like Res1 and suppressed the cold-sensitive phenotype of *rep2*⁻ cells, but not the meiotic defect of *res2*⁻ cells. Chimeras between Res1 and Res2 made by splicing at the end of the second ankyrin motif produced similar results. The functional difference between Res1 and Res2 is almost exclusively determined by the C-terminal half of the two molecules which contains domains essential for their function.

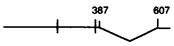
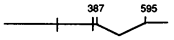
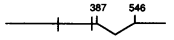
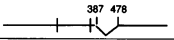
A second set of chimeras in which C-terminal 10–91 amino acids were swapped between Res1 and Res2 could rescue *res1*⁻ cells to a similar level, but they differed in their ability to suppress *rep2*⁻ and *res2*⁻ cells (Table 5). By increasing the length of the swapped sequences, Res1 rapidly lost the ability to act as Res1 and gradually gained the ability to act as Res2, whereas Res2 gradually lost the ability to act as Res2 and gained the ability to act as Res1.

Requirement of the Ankyrin Repeats for Mitotic and Meiotic Function

The ankyrin motif is among the most conserved sequences of the SWI/Cdc10 family members (Koch *et al.*, 1993). *res1*⁺ and *res2*⁺ were deleted for an internal region of varying length involving the two ankyrin motifs and their intervening sequence and overexpressed from the SV40 promoter in *res1*⁻, *rep2*⁻, and *res2*⁻ cells (Table 6). All of these truncated Res1 and Res2 molecules suppressed *res1*⁻ cells and retained their specific function.

An internally truncated Res2 molecule consisting of the C-terminal 257 and N-terminal 170 amino acids was significantly reduced in its ability to rescue *res1*⁻ cells. Replacement of the N-terminal 170 amino acids with the corresponding N-terminal 159 amino acids of Res1 failed to restore the full mitotic activity of the molecule. A similar internally truncated Res1 molecule could suppress the cold-sensitive phenotype of *res1*⁻ cells, and replacement of its N-terminal 159 amino acids with the corresponding N-terminal domain of Res2 did not alter the mitotic activity of the molecule.

Table 4 Deletion analysis of Res1

Construct	% Suppression of rep2⁻	% Asci 4 spores	% Suppression of res1⁻	Suppression of res1⁻ rep2⁻
pcLx empty vector	0	3 ± 1	0	no
pcLres1	86 ± 5	1 ± 0.5	97 ± 4	yes
−14AA	18 ± 8	2 ± 1	90 ± 4	yes
−45AA	10 ± 3	4 ± 2	66 ± 4	yes
−162AA	2 ± 1	8 ± 3	13 ± 8	yes
−250AA	3 ± 2	6 ± 4	54 ± 6	no
−334AA	<1	9 ± 2	<1	no
−378AA	<1	5 ± 1	3 ± 1	no
	25 ± 9	3	62 ± 1	—
	42 ± 14	2	65 ± 5	—
	60 ± 8	4	69 ± 7	—
	78 ± 6	1	73 ± 14	—

Representation of Res1 and Res2 and use of symbols is the same as described.

Table 5. Functional analysis of chimeric molecules between Res1 and Res2

Construct	% Asci with 4 spores	% Suppression of rep2⁻ cells	% Suppression of res1⁻ cells
pcLX empty vector	7 ± 2	<1	<1
pcLres1	2 ± 1	81 ± 6	94 ± 4
pcLres2	67 ± 3	<1	74 ± 11
303AA 341AA	50 ± 1	5 ± 4	25 ± 1
314AA 332AA	12 ± 4	40 ± 23	64 ± 7
387AA 257AA	56 ± 3	12 ± 4	93 ± 1
398AA 248AA	25 ± 4	88 ± 6	91 ± 5
238 AA 257AA	50 ± 3	7	64 ± 8
623AA 10AA	2 ± 1	35 ± 4	84 ± 8
604AA 30AA	6 ± 1	19 ± 3	89 ± 0.3
546AA 74AA	7 ± 1	3 ± 2	88 ± 4
645AA 12AA	37 ± 3	38 ± 11	90 ± 6
626AA 31AA	33 ± 6	57 ± 11	98 ± 5
581AA 91AA	7 ± 3	70 ± 20	92 ± 4

Chimeric constructs are composed of light and dark horizontal lines. The number above the horizontal lines in each construct corresponds to the number of amino acids originating from either Res1 or Res2.

These internally truncated Res1 molecules lacking a domain between amino acids 159–226 are structurally Res1-like, but functionally Res1-Res2 chimeras. They were fully active as start molecules for the mitotic cycle and partially suppressed the meiotic defect in *res2*⁻ cells.

C-terminal 41 Amino Acids Restrict Res2 to Require Rep2 for the Start Function in the Mitotic Cycle

In the mitotic cycle, Rep2 is required for the activation of the Res2-Cdc10 complex (Nakashima *et al.*, 1995). *rep2*⁻ and *rep2*⁻*res1*⁻ cells were used to specifically detect Res1-like mitotic activity. Unlike Res1, in these host cells Res2 has no apparent cell cycle start activity because Rep2 is required for the mitotic function of the Res2-Cdc10 complex. In the absence of Rep2, Res2 is unable to suppress the cold-sensitive growth defect in *rep2*⁻ cells.

Some C-terminal truncated *res2*⁺ constructs were found to suppress the cold sensitivity of *rep2*⁻ cells (Table 2). When 12 amino acids were removed from the C-terminus, Res2 started to suppress the *rep2*⁻ host. Further deletion of up to 41 amino acids generated a Res2 molecule half as active as Res1. This same molecule can still suppress the meiotic defect in *res2*⁻ cells, though reduced. Truncated molecules with deletions up to 76 amino acids from the C-terminus behaved like Res1 and Res2 and suppressed the cold-sensitive phenotype of *res1*⁻*rep2*⁻ double-disruptant cells and the meiotic defect in *res2*⁻ cells.

Domain Essential for Binding of Rep2 to Res2 In Vitro

Res2 but not Res1 can bind to Rep2 in vitro (Nakashima *et al.*, 1995). To locate this binding site in Res2 and to determine its biological significance, we performed a Rep2-Res2 in vitro binding assay with various deletion mutants of Res2 and its chimeras with Res1 (Table 7 and Figure 2). Deletions up to 259 amino acids from the C-terminal end of the Res2 molecule did not affect the interaction of Res2 with GST-Rep2 (Table 7 and Figure 2). Smaller Res2 proteins with C-terminal deletions of 419 amino acids showed no significant binding to GST-Rep2 (our unpublished data). Similar results were obtained with chimeras between Res2 and Res1. A chimeric Res2-Res1 molecule composed of the N-terminal 398 amino acids of Res2 and C-terminal 248 amino acids of Res1 bound to GST-Rep2 very well, whereas the opposite chimeric construct interacted only very weakly with GST-Rep2 (Table 7 and Figure 2). Internally truncated Res2 molecules consisting of the N-terminal 170 amino acids and C-terminal 257 amino acids of Res2 as well as chimeric Res2-Res1 molecules consisting of the N-terminal 170 amino acids of Res2 and the C-terminal domain of Res1 still bound GST-Rep2; however, in a reduced manner.

Table 6. Requirement of the ankyrin repeats for mitotic and meiotic function

Construct	% Asci with 4 spores	% Suppression of rep2⁻ cells	% Suppression of res1⁻ cells
pcLX empty vector	5 ± 2	<1	<1
pcLres1	1 ± 0.5	82 ± 13	93 ± 3
pcLres2	68 ± 4	<1	72 ± 9
−ANK 238AA 257AA	65 ± 3	15 ± 5	62 ± 12
170AA 257AA	48 ± 1	<1	19 ± 12
159AA 257AA	46 ± 2	<1	21 ± 18
−ANK 226AA 248AA	11 ± 1	87 ± 5	95 ± 12
159AA 248AA	27 ± 3	78 ± 8	90 ± 6
170AA 248AA	24 ± 2	80 ± 4	84 ± 11

Representation of Res1 and Res2 and use of symbols is the same as described. The numbers above the lines correspond to the number of amino acids in the depicted fragment and ANK indicates the ankyrin motif.

Table 7. In vitro Rep2 binding assay

Construct	In vitro binding to GST-Rep2 fusion protein	% Suppression of res1⁻ cells
Res1	+/−	91 ± 12
Res2	+++	75 ± 12
−12AA	++/+++	93 ± 2
−32AA	++/+++	94 ± 4
−259AA	++/+++	<1
−343AA	++	<1
−ANK	++	62 ± 12
170AA 257AA	+	19 ± 12
170AA 248AA	+	84 ± 11
626AA 31AA	+++	98 ± 5
497AA 160AA	+++	83 ± 10
398AA 248AA	+++	91 ± 5
387AA 257AA	+/−	93 ± 1

For comparison, the right column summarizes the mitotic ability of each construct tested as determined by the suppression of the cold-sensitive phenotype of the *res1*⁻ host.

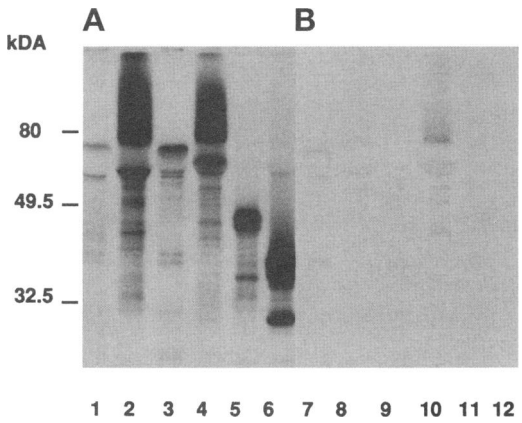


Figure 2. Rep2 binds to Res2 and various Res2 derivatives in vitro. [^{35}S]methionine-labeled Res1, Res2, chimeric molecules, and C-terminal truncated Res2 were precipitated with bacterially produced GST-Rep2 bound to glutathione-Sepharose beads (A) or glutathione-Sepharose beads alone (B), separated in SDS-polyacrylamide gels, and autoradiographed. Lanes 1 and 7, Res1; lanes 2 and 8, Res2; lanes 3 and 9, chimeric Res1-Res2, N-terminal 387 amino acids of Res1 and C-terminal 257 amino acids of Res2; lanes 4 and 10, chimeric Res2-Res1, N-terminal 398 amino acids of Res2 and C-terminal 248 amino acids of Res1; lanes 5 and 11, C-terminal 259 amino acids deleted from Res2; lanes 6 and 12, C-terminal 343 amino acids deleted from Res2.

DISCUSSION

The fission yeast cell cycle start molecules Res1 and Res2 are structurally homologous and functionally partly overlapping, yet they participate in two distinct cellular processes. Res1 functions exclusively in the mitotic cycle, whereas Res2 plays a major role in the meiotic cycle in *S. pombe*. The two molecules are most homologous in their N-terminal conserved region, representing the DNA-binding domain and the two centrally located ankyrin motifs (Figure 1). Both of these structural motifs are also shared with the *S. cerevisiae* proteins SWI4 and Mbp1. Res1 and Res2 differ the most in the C-terminal portion which is very heterogeneous between the two proteins (Figure 1). In this article, we have identified several discrete domains in the C-terminal region of Res1 and Res2 which are essential for their functions and responsible for the functional specificity of the two molecules. The positioning of the domains and their tentative functional assignments were done based on the data presented and are illustrated in Figure 3. In addition, our data suggest that, if overexpressed, the conserved ankyrin motif may not be absolutely required for the mitotic or meiotic function of either Res1 or Res2 and may be potentially dispensable. The functional specificity of Res1 and Res2 is independent of the N-terminal conserved domain and the conserved ankyrin motifs in the center of each molecule. Thus, functional specificity of Res1 and Res2 as demonstrated by chimeric molecules is exclusively determined by the C-terminal portion of either molecule.

Res2 contains a domain(s) in the C-terminal 217 amino acids that is required for its mitotic function. This conclusion is based on the results obtained with different truncated Res2 molecules overexpressed in

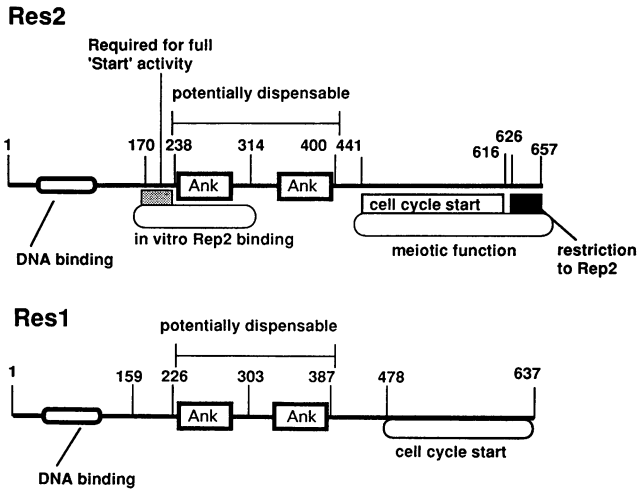


Figure 3. Localization of functional domains in Res1 and Res2. The Res1 and Res2 molecules are represented by a horizontal line with the number 1 indicating the first amino acid of the ORF and 637 or 657, respectively, the last one before the stop codon. Vertical lines with number indicate the position of amino acids.

res1$^-$, *rep2*$^-$, and *res1*$^-$*rep2*$^-$ cells. The domain stretches from amino acids 441–616. In the mitotic cycle, Rep2 is the major transactivator of the Res2-Cdc10 complex (Nakashima *et al.*, 1995). Res2 contains a domain of approximately 31 amino acids at the C-terminus that restricts the molecule to depend on Rep2 in the mitotic cycle (Table 2 and Figure 3). Deletion of this domain converts Res2 to a functional chimera having the properties of both Res2 and Res1. A domain of approximately 180 amino acids that is linked to this small restriction domain is essential for Res2 function. Deletion of either parts of or the entire domain results in Res2 molecules with impaired mitotic and meiotic function (Tables 2 and 3). It is possible that this domain might be required for functional interaction with transactivator subunits, including Rep2 and the conserved N-terminal DNA-binding domain. This domain may also be involved in interaction with Cdc10 since it roughly corresponds to the region in Res1 that has been shown to bind to the Cdc10 molecule in vitro (Ayte *et al.*, 1995). Nevertheless, the requirement for this domain in Res2 is independent of its possible interaction with Cdc10 because overproduction of the Res2 molecule can suppress the mitotic and meiotic defects of a *cdc10* null allele mutant cell (Sturm and Okayama, unpublished data).

Res2 contains a domain just upstream of the first ankyrin motif, which spans from amino acids 170–238 and is indispensable for full Res2 activity in the mitotic cycle (Table 6). Because this domain is located within the in vitro Rep2-binding region and is required for the mitotic activity of the C-terminal domains of Res2, its role may be to provide an effective site for interaction with or binding of Rep2.

Unlike Res1, Res2 has a unique meiotic function. The domain required for this meiotic function stretches from the C-terminal end to amino acid 441 and is partially overlapping with the one required for the mitotic role of the protein (Tables 2, 3, and 5; Figure 3). However, at present it is unknown how this domain exerts meiotic function and whether the same sequence in this domain is responsible for both mitotic and meiotic functions. As experimental results with chimeras between Res1 and Res2 indicate, the meiotic function of Res2 is exclusively determined by this domain and is independent of the conserved N-terminal DNA-binding domain. A Res1-Res2 chimeric molecule consisting of the C-terminal 257 amino acids of Res2 and the N-terminal 387 amino acids of Res1 suppresses the meiotic defect in *res2*$^-$ cells almost as well as wild-type Res2. The mechanism by which Res2 executes its meiotic function may radically differ from the one by which it executes its cell cycle start function.

We determined at least one major domain in the C-terminal region of Res1, which is essential for the protein to execute its start function in the mitotic cell cycle. This domain spans from amino acids 478 to the C-teminus and does not require interaction with Rep2 (Table 4), but most likely other components essential for cell cycle start.

Unlike Res2, wild-type untruncated Res1 itself has no detectable meiotic function as determined by its inability to suppress the meiotic defect in *res2*$^-$ cells. On the contrary, the Res1 molecule exerts an inhibitory effect, reducing the number of zygotes which will form asci with four spores below background levels. Deletion of the two copies of the conserved ankyrin motifs and sequences adjacent to these at the N-terminal end removes this inhibition and these internally truncated Res1 proteins can at least partially suppress the meiotic defect in *res2*$^-$ cells. We have to conclude that in the C-terminal part of Res1 in addition to domains essential for its mitotic function a domain(s) is located with a potential meiotic function. However, because of the presence of the ankyrin motifs and adjacent sequences at the N-terminal end, this potential meiotic function of the protein is completely blocked. The possibility of generating chimeric molecules which structurally resemble either one of the two proteins, but have the functional properties of Res1 as well as Res2, raises an interesting question regarding the evolutionary origin of the two molecules. One might speculate that the ancestral molecule very likely had the functional properties of both proteins and that evolutionary events took place which separated both functions onto two proteins.

The ankyrin motifs are among the most conserved sequences in the Swi/Cdc10 family of transcription factors. Overexpression of *res1*$^+$ and *res2*$^+$ lacking the ankyrin repeats from the SV40 promoter (Table 4) and analysis of these truncated proteins in *res1*$^-$, *res2*$^-$, or *rep2*$^-$ cells suggested that under these particular conditions this motif is not absolutely required for the functional specificities of the two proteins and for the biological activity in general. However, it is difficult to infere from these results unambiguously the dispensability of the ankyrin repeats under physiological conditions. Moreover, it is also possible that this motif is involved either directly or indirectly in other aspects of the start event which we were unable to detect under the experimental conditions we used.

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