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Analysis of the Cdc28 protein kinase complex by dosage suppression

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Summary

In the interest of identifying components of the Cdc28 protein kinase complex, dosage suppression analysis was performed on temperature-sensitive and dominant negative *CDC28* mutations. Dosage suppression is based on a rationale in which elevated expression of wild-type genes can rescue mutations in a target gene as a result of interaction between the respective encoded proteins. Three sequences capable of rescuing a temperature sensitive *cdc28* mutation were isolated from a library of wild-type genomic DNA segments in the high copy vector YEp13. Two of these, named *CLN1* and *CLN2* were found to encode closely related proteins with homology to cyclins. The third, *CKS1*, encodes an 18K ($K=10^3 M_r$) protein that has been shown to be a component of the Cdc28 protein kinase complex and is a homolog of the *suc1*$^+$ product of fission yeast. A number of dosage suppressors of the *CDC28-dn1* dominant negative mutation have been isolated. The one analyzed to date encodes a truncated subunit of the mitochondrial enzyme succinyl-CoA synthetase. The basis for suppression in this case remains to be elucidated.

Introduction

Mutational analysis of the cell division cycle in yeast has led to the identification and isolation of genes important for cell cycle control (Hartwell *et al.* 1974; Nurse *et al.* 1976). The product of the gene *CDC28*, required for cell cycle initiation in budding yeast, *Saccharomyces cerevisiae*, is a protein kinase that is regulated by both intracellular and extracellular signals relevant to division control (Lörincz and Reed, 1984; Reed *et al.* 1985; Mendenhall *et al.* 1987). Homologs of the Cdc28 protein kinase have been found in a variety of eukaryotic species, including man, where a role in cell cycle control, if not yet proven, is strongly suggested (Simanis and Nurse, 1986; Lee and Nurse, 1987; Brizuela *et al.* 1987; Gautier *et al.* 1988; Dunphy *et al.* 1988; Labbe *et al.* 1988; Arion *et al.* 1988). In all cases, the active forms of the protein kinase exist as multimeric complexes containing polypeptides in addition to p34, the product of *CDC28* and its homologs (Wittenberg and Reed, 1988; Draetta and Beach, 1988; Gautier *et al.* 1988; Dunphy *et al.* 1988). Furthermore, assembly and disassembly of complexes is responsive to both intracellular and extracellular conditions affecting cell cycle progression (Wittenberg and Reed, 1988; Draetta and Beach, 1988). Thus, it appears in yeast as well as in man that a highly conserved

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protein kinase central to cell cycle control is regulated, at least in part, at the level of assembly and disassembly of a multiprotein complex. The goal of the work described here is to use genetic methods to identify other components of the Cdc28 protein kinase complex in *S. cerevisiae*. It is assumed, due to the structural and functional homology observed between the Cdc28 protein kinase and its homologs across species lines, that the compositions and dynamics of the active complexes will be highly conserved.

Materials and methods

Isolation of dosage suppressors

Isolation of dosage suppressors of temperature-sensitive *cdc28* mutations was as described by Hadwiger *et al.* (1989*a*,*b*). A library of yeast wild-type DNA segments in the multicopy vector YEp13 (Broach *et al.* 1979; Nasmyth and Tatchell, 1980) was transformed by the spheroplast method (Hinnen *et al.* 1978) into strain JF210-92 carrying the ts (temperature-sensitive) mutation *cdc28-4* (Reed, 1980). Based on restriction analysis and hybridization analysis, three different sequences, in addition to the *CDC28* gene itself, were able to rescue the *cdc28-4* ts mutation as well as two other alleles tested, *cdc28-9* and *cdc28-13*.

Dosage suppressors of mutation *CDC28-dn1* (Mendenhall *et al.* 1988) were isolated by transforming strain MDMY358 (Mendenhall *et al.* 1988) containing the dominant negative gene under control of the glucose-repressible/galactose-inducible *GAL1* promoter with the same library in YEp13. Transformant colonies were initially grown on medium containing glucose and then replica-plated to medium containing galactose. Plasmids which permitted growth on galactose were analyzed.

Subcloning and sequence analysis

Subcloning of suppressor sequences was performed by isolating progressively smaller restriction fragments from the initial clones and reinserting them in multicopy yeast plasmids for rescreening. Suppressor coding regions were localized by insertional mutagenesis of subcloned sequences. Restriction fragments containing selectable yeast markers were inserted into convenient restriction sites within subcloned sequences and loss of suppression scored. These insertion sites then served as starting points for DNA sequence analysis. Phagemid vectors (Pharmacia) and the dideoxy method of Sanger *et al.* (1978) were employed. Protein sequence comparisons and data base screens were performed using programs and facilities available through BIONET.

Chromosomal insertional mutations (disruptions)

Chromosomal gene disruptions were performed using insertionally-mutated coding regions generated as described above. One-step gene replacement (Rothstein, 1983) allowed replacement of one wild-type allele of a diploid with the insertionally mutated sequence to form a heterozygote. After confirmation by Southern blot (Southern, 1975), heterozygous diploids were sporulated and subjected to tetrad analysis (Mortimer and Hawthorne, 1969) in order to assess the phenotype of disruption.

Results and discussion

Dosage suppressors of cdc28 *mutants*

Three different non-*CDC28* sequences capable of rescuing the *cdc28-4* temperature-sensitive mutation were isolated from a yeast genomic library based on the multicopy vector, YEp13 (Broach *et al.* 1979; Nasmyth and Tatchell, 1980). These were initially designated *PSC1*, *PSC2* and *PSC3* for Plasmid Suppressor of *cdc28*. All

three suppressors raised the restrictive temperature of mutant cells approximately 2°C. None was, however, capable of rescuing a null allele of *cdc28*, indicating that suppression does not occur by bypassing the *CDC28* function.

A number of dosage suppressors of the dominant negative allele *CDC28-dn1* were isolated (Mendenhall *et al.* 1988). One of these, designated *PSC4*, has been subjected to further study. *PSC4* was found to be distinct from *PSC1*, *PSC2* and *PSC3* and was incapable of rescuing temperature-sensitive mutations. Conversely, *PSC1*, *PSC2* and *PSC3* were incapable of rescuing the dominant negative *CDC28-dn1* mutation.

Subcloning and sequencing of dosage suppressors

Suppressing sequences were subcloned from plasmid inserts to smaller restriction fragments. Internal restriction sites were then used as points for insertional mutation by inserting selectable marker genes. Such constructs were scored for loss of ability to suppress in order to assess whether the suppressor coding region had been interrupted. DNA sequence analysis was then performed bidirectionally from this point of insertion. In each case an open reading frame was found to flank the point of insertion, consistent with the results of the mutational analysis. The open reading frames corresponding to *PSC1* and *PSC2* were found to encode polypeptides of 546 and 545 amino acids, respectively. That corresponding to *PSC3* encodes a polypeptide of 150 amino acids. For reasons that will be discussed below, it was impossible to determine the precise number of amino acids contained in the protein encoded by *PSC4*.

When compared to each other, the predicted products of *PSC1* and *PSC2* were found to be highly homologous at the level of primary structure (Fig. 1). Over the entire sequences, a 57% identity was found. However, the predicted proteins were considerably more highly conserved in their aminoterminal 50%, with amino acid identities found at 72% of the positions. This high degree of homology suggests that Psc1 and Psc2 have similar, if not identical, functions. When these sequences were compared to the data bases of known and predicted protein sequences, homology was found to a family of proteins known as cyclins. Cyclins were originally discovered in the oocytes and embryos of marine invertebrates, where they undergo dramatic fluctuations in level as a function of progression through the cell cycle (Evans *et al.* 1983; Rosenthal *et al.* 1980). It has been proposed that cyclins are rate-limiting regulators of cell cycle events based on their kinetics of accumulation and degradation and on the ability of injected cyclin mRNA to cause *Xenopus* oocytes arrested in meiotic prophase to undergo maturation (Swensen *et al.* 1986). The isolation, therefore, of genes encoding cyclin-like proteins as suppressors of *cdc28* mutations is not surprising in light of the role of the Cdc28 protein kinase in control of cell cycle progression in yeast. Because of the homology to cyclins, *PSC1* and *PSC2* were renamed *CLN1* and *CLN2*, respectively. In fact, the homology is limited to approximately 150 amino acids, sometimes referred to as the 'cyclin box', since it is the only conserved region when comparing diverse cyclins. A comparison of the

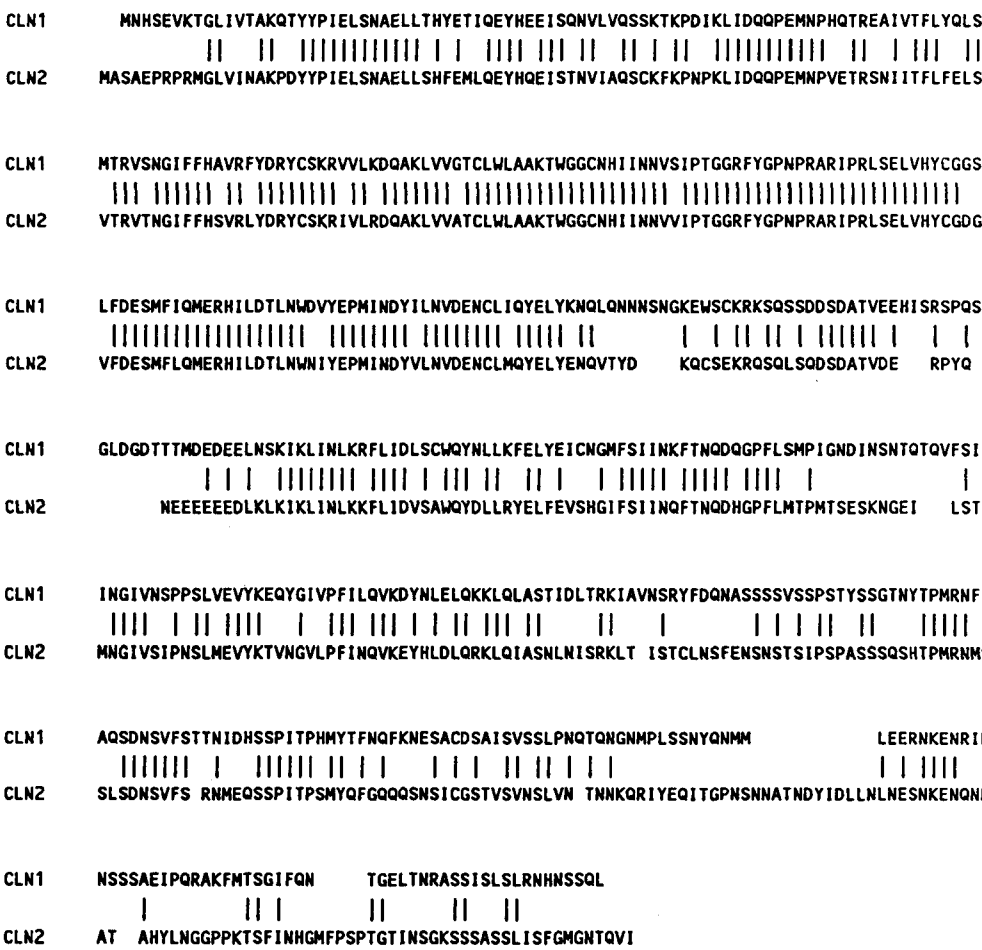

Fig. 1. Comparison of the predicted products of *CLN1* and *CLN2*. The proteins predicted from DNA sequence analysis of *CLN1* and *CLN2* were aligned for maximal homology. The vertical bars indicate amino acid identities.

cyclin box region of Cln1 with that of Cyclin A from clam (Swensen *et al.* 1986) is shown in Fig. 2.

The polypeptide predicted by the DNA sequence of *PSC3* showed strong homology with the *suc1*$^+$ product of fission yeast *Schizosaccharomyces pombe* (Hayles *et al.* 1986; Hindley *et al.* 1987; Fig. 3). Alignment of the two sequences gives identities at 67 % of amino acid positions. The *PSC3* product is extended at the carboxyterminus to give a molecular weight of approximately 18K (K=$10^3 M_r$) rather than 13K, the predicted molecular weight for the *suc1*$^+$ product (Hadwiger *et al.* 1989*a*). Interestingly, the *suc1*$^+$ gene was isolated as a dosage suppressor of mutations in the *cdc2* gene, the *S. pombe* homolog of *CDC28*. As is the case with the *suc1*$^+$ gene product and the cdc2 protein kinase of *S. pombe* (Brizuela *et al.* 1987), the *PSC3* gene product was shown to be a component of the active Cdc28

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CLN1  12  VTAKQTYYPIELSNAELLTHYETIQEYHEEISQNVLVQSSKTKPDIKLID
          ||    | |   | |  || || |||  |    | ||   |
CYCA 138  ESPMILDLPEEEKPLDREAVILTVPEYEEDIYNYLRQAEMKNRAKPGYMK

CLN1  62  QQPEMNPHQTREAIVTFLYQLSVMTRVSNGIFFHAVRFYDRYCSKRVVLK
          |||| |  | || || |||  ||    | ||  |||  | | |
CYCA 189  RQTDI TTSMRCILVDWLVEVSEEYKLHRETLFLGVQLIDRFLVDHAVSK

CLN1 112  DQAKLVVGTCWLAAKTWGGCNHIINNVSIPTGGRFYGPNPRARIPRLSEL
            || | |||||       | | |  |   ||||  || ||
CYCA 238  GKLQLVGVTAFLAAKYEEIYPPDVKEFAYITDDT YTSQQVLRMEHLILK

CLN1 162  VHYCGGSDLFDESMFIQMERHILDTLNWDVYEPMINDYILNVDENCLIQY
          |   |  | | |  | || |    ||   | || | | |
CYCA 287  VLTFDVAVPTTNW FCEDFLKSCDADDKLKSLTMFLTELTLIDMDAYLKY
```

Fig. 2. Comparison of the 'cyclin box' regions of the *CLN1* product (residues 12–212) and of cyclin A from clam (residues 138–338; Swensen *et al.* 1986). The vertical bars indicate amino acid identities or conservative substitutions.

protein kinase complex (Hadwiger *et al.* 1989*a*). Therefore, the gene was renamed *CKS1*, for Cdc28 Kinase Subunit. This result confirms that the approach of dosage suppression can identify elements that interact directly with the primary element targeted by mutation. It remains to be determined whether Cln1 and Cln2 interact directly with the Cdc28 protein kinase as well.

The polypeptide predicted by the suppressor *PSC4* was found to share homology with the α subunits of the tricarboxylic acid cycle enzyme succinyl-CoA synthetase of *Escherichia coli* and vertebrates, respectively (Fig. 4). 52 % identity was found with the *E. coli* sequence (Buck *et al.* 1985) while 60 % identity was found with the rat liver sequence (Henning *et al.* 1988). It is assumed that *PSC4* corresponds to the α subunit of succinyl-CoA synthetase of yeast. However, *PSC4* encodes a polypeptide shorter than expected and which contains an incomplete mitochondrial import sequence, essential for transport into the mitochondrial matrix (van Loon *et al.*

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Cks1  MYHHYHAFQGRKLTDQERARVLEFQDSIHYSPRYSDDNYEYRHVMLPKAMLKVIPSDYFNSEVGTLRILTEDEWR
      |        | ||  || |  | | |||||||| || |||||||||||||| || |||| | |||||| | |||
Suc1  M SK SG VPRLLTASERERLEPFIDQIHYSPRYADDEYEYRHVMLPKAMLKAIPTDYFNPETGTLRILQEEEWR

Cks1  GLGITQSLGWEHYECHAPEPHILLFKRPLNYEAELRAATAAAQQQQQQQQQQQQQQQQQHQTQSISNDMQVPPQIS
      ||||||||||| || | ||||||||||  |          ||
Suc1  GLGITQSLGWEMYEVHVPEPHILLFKREKDYQ  MK  FS  QQRGG
```

Fig. 3. Comparison of the predicted product of *CKS1* and the *suc1*$^+$ product of the fission yeast *Schizosaccharomyces pombe* (Hayles *et al.* 1986; Hindley *et al.* 1987). The vertical bars indicate amino acid identities.

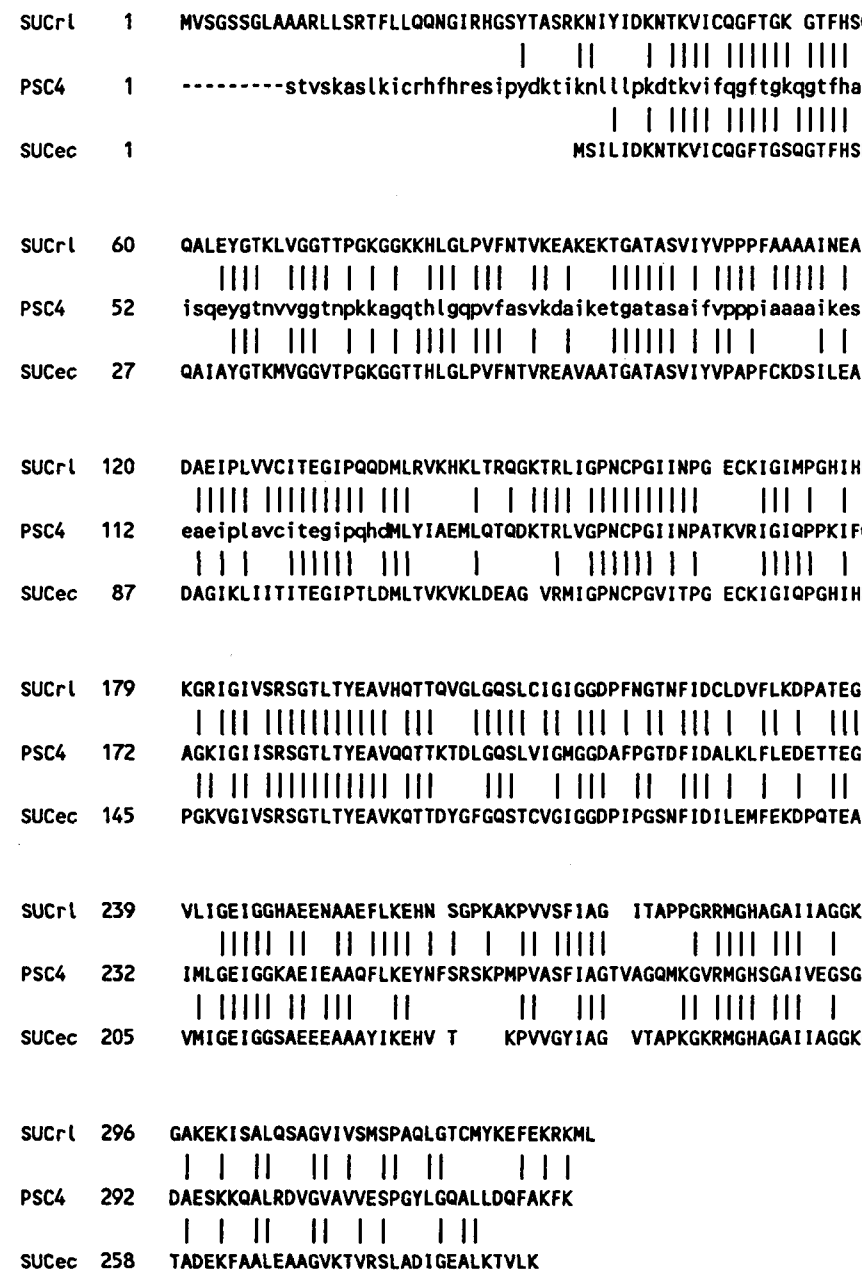

Fig. 4. Comparison of the predicted product of the *PSC4* gene and succinyl-CoA synthetase α subunits from *E. coli* (SUCec; Buck *et al.* 1985) and rat liver (SUCrl; Henning *et al.* 1988). The vertical bars indicate amino acid identities between the Psc4 sequence and either one of the succinyl-CoA synthetases. The lower case letters and dashed line at the aminoterminal end of the Psc4 sequence indicate that the initiation codon utilized is not known and may be upstream within vector sequences. Upper case letters begin at the first in-frame methionine codon within the Psc4 sequence.

1986). Isolation of a larger segment from the genomic region surrounding *PSC4* indicates, however, that the suppressor was an aminoterminally-truncated version of the gene (missing the first three codons; data not shown). It is not certain whether translation begins at an internal methionine or at a methionine encoded by vector sequences (Fig. 4). The full length gene encodes a polypeptide with all of the properties expected of succinyl-CoA synthetase, including a mitochondrial import consensus sequence (data not shown). The reason for suppression of a dominant negative mutation of *CDC28* by a truncated allele of succinyl-CoA synthetase is obscure. The α subunit of succinyl-CoA synthetase has some homology to primitive protein kinases involved in prokaryotic signal transduction (Hess *et al.* 1987; Ninfa *et al.* 1988; M. Simon, personal communication) and one possible mechanism might be assumption of a novel protein kinase activity by this suppressor. On the other hand, the primary structure of the *PSC4* product reveals some homology to conserved regions of the Cdc28 polypeptide which may be targets of negative regulation (data not shown). Thus *PSC4* may suppress by titrating a negative regulatory element. It is noteworthy that the full length gene does not suppress as effectively as the truncated allele. The reason may be that cytoplasmic accumulation, caused by removal of mitochondrial import sequences of the truncated allele, may be important for suppression. Alternatively, truncation, especially if initiation of translation occurs at the first internal methionine, may lead to a protein with drastically altered properties.

Mutant phenotypes associated with dosage suppressor genes

Insertional mutation of either *CLN1* or *CLN2* conferred no obvious phenotypes. However, the double mutant, although viable, showed impaired growth and severe morphological phenotypes. Mutant cells are abnormally large and mis-shapen, indicative of a cell division cycle defect. The interaction of *cln1* and *cln2* mutations is consistent with the notion, based on sequence homology, that the *CLN1* and *CLN2* products provide overlapping or identical functions. The non-lethal phenotype conferred by the double mutant suggests that another gene product is able to provide cyclin function in *S. cerevisiae*. A potential candidate is the product of a gene known alternately as *DAF1* (Cross, 1988) or *WHI1* (Sudbery *et al.* 1980; Nash *et al.* 1988). Mutations in this gene confer dominant cell cycle advance phenotypes, suggesting that the product is rate-limiting for cell cycle progression. The *DAF1/WHI1* product is a cyclin-like protein with only minimal structural homology to the *CLN1* and *CLN2* products. However, mutations in *CLN1* and *CLN2* confer similar dominant mutations to *DAF1/WHI1* mutations (Hadwiger *et al.* 1989*b*; unpublished observations), suggesting that all three gene products may be functionally homologous.

Insertional mutation of the *CKS1* gene is lethal (Hadwiger *et al.* 1989*a*). Such mutants can be propagated by providing the wild-type gene on a plasmid. When such a plasmid is mitotically unstable, segregation of the wild-type gene with high frequency gives rise to a population cells showing a cell division cycle phenotype characteristic of cells arrested in G_1 (Hadwiger *et al.* 1989*a*). Thus, it appears that the *CKS1* product is essential for G_1 functions of the Cdc28 protein kinase. This

observation is in contrast with the suggestion that the *suc1*$^+$ product of *S. pombe* is an inhibitor of the cdc2 protein kinase (P. Nurse, personal communication).

Insertional mutation of *PSC4* conferred no cell cycle phenotype. Nor were any respiratory defects observed in mutant cells, as expected for elimination of a tricarboxylic acid cycle enzyme. We conclude that multiple genes must encode the α subunit of succinyl-CoA synthetase and that elimination of one is not sufficient to confer a respiratory phenotype. The observation that labelled *PSC4* DNA reacts with a heterologous sequence on low-stringency blots of genomic yeast DNA (Southern, 1974) is consistent with this.

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