Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast CDC34 (UBC3) ubiquitin conjugating (E2) enzyme

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The ubiquitin conjugating (E2) enzyme encoded by CDC34 (UBC3) in *Saccharomyces cerevisiae* is required for the G₁ to S transition of the cell cycle. CDC34 consists of a 170 residue amino-terminal domain that is homologous to that found in other E2s, followed by a 125 residue carboxyl-terminal domain that is specific to CDC34. We found that a truncation mutant of CDC34 which lacked the CDC34 carboxyl-terminal domain could not support the essential function of CDC34 in the cell cycle *in vivo*. To explore further the role of the carboxyl-terminal domain in determining the cell cycle function of CDC34, we constructed and characterized genes encoding chimeric E2s incorporating sequences from CDC34 and the related but functionally distinct E2 RAD6 (UBC2). We found that a construct encoding a chimeric RAD6–CDC34 ubiquitin conjugating enzyme, in which the 21 residue acidic carboxyl-terminal domain of RAD6 has been replaced with the 125 residue carboxyl-terminal domain of CDC34, performed the essential functions of CDC34 *in vivo*. This chimeric E2 also complemented the growth deficiency, UV sensitivity and sporulation deficiency of *rad6* mutant strains. Deletion analysis of the CDC34 carboxyl-terminal domain in both CDC34 and the RAD6–CDC34 chimeric E2 identified a region comprising residues 171–244 of CDC34 that was sufficient to confer CDC34 function on the amino-terminal domains of CDC34 and RAD6. We suggest that this region interacts with substrates of CDC34 or with *trans*-acting factors (such as CDC34-specific ubiquitin protein ligases) that govern the substrate selectivity of CDC34. Congruent results demonstrating a positive role for the carboxyl-terminal domain of CDC34 in the essential function of CDC34 have also been obtained by Silver *et al.* (1992) and are reported in the accompanying paper.

Key words: CDC34 (UBC3)/cell cycle/RAD6 (UBC2)/ubiquitin

Introduction

Covalent attachment of the highly conserved 76 residue protein ubiquitin to other eukaryotic proteins is implicated in cellular processes as seemingly diverse as selective protein

turnover, cell cycle progression, DNA repair and the stress response (reviewed in Finley and Chau, 1991). The many functions of protein ubiquitination are governed by a multiplicity of ubiquitin conjugating (or E2) enzymes (Pickart and Rose, 1985; Haas and Bright, 1988; Haas *et al.*, 1988; Pickart and Vella, 1988; Jentsch *et al.*, 1990, 1991). E2 enzymes facilitate the transfer of ubiquitin from its activated form as a thiol ester with ubiquitin activating (or E1) enzyme to other cellular proteins via the intermediate formation of a ubiquitin thiol ester with the E2 enzyme (Hershko *et al.*, 1983; Pickart and Rose, 1985; Haas *et al.*, 1988). Different E2 enzymes can act in distinct cellular functions, apparently reflecting differences in their substrate selectivity (Jentsch *et al.*, 1991). *In vitro*, E2s catalyze ubiquitination either alone or in concert with other factors called ubiquitin protein ligases (or E3s), which regulate the substrate specificity of ubiquitination in those cases where they are required (Hershko *et al.*, 1986; Reiss *et al.*, 1988). All E2s have a homologous amino-terminal (N-terminal) sequence domain containing a conserved cysteine residue where the thiol ester with ubiquitin is formed. E2s have been classified into two types (Jentsch *et al.*, 1990): class I E2s consist solely of this N-terminal domain, whereas class II E2s also bear a carboxyl-terminal (C-terminal) extension. Jentsch *et al.* (1990) have suggested that the C-terminal domains may specify the substrate selectivity of the class II E2s, whereas the class I E2s may require exogenous factors (such as E3 proteins) for substrate selection.

In *Saccharomyces cerevisiae*, genes encoding at least nine different E2 enzymes have been identified (Jentsch *et al.*, 1991; Qin *et al.*, 1991). Genes that encode two of the yeast E2s, RAD6 and CDC34, were first identified in other contexts. RAD6, now also referred to as UBC2 (Jentsch *et al.*, 1987), is required for a number of cellular functions including post-replication repair, sporulation and induced mutagenesis (Prakash *et al.*, 1990). RAD6 also functions in the N-end rule pathway of selective proteolysis (Dohmen *et al.*, 1991; Sung *et al.*, 1991) and may play a role in cell cycle progression sometime during the transit through the S or G₂ phases of the cell cycle (Ellison *et al.*, 1991). CDC34, now also referred to as UBC3 (Goebel *et al.*, 1988), is required for the transition from the G₁ to S phases of the cell cycle and was first recognized as encoding an E2 enzyme by virtue of its homology to RAD6. Both CDC34 and RAD6 encode class II E2s that catalyze the conjugation of ubiquitin to histones *in vitro*, and both require the presence of their (acidic) C-terminal extension for efficient histone conjugation activity *in vitro* (Jentsch *et al.*, 1987; Goebel *et al.*, 1988; Sung *et al.*, 1988). However, their relevant *in vivo* substrates have not been identified. A truncation mutant of RAD6 that lacks the 23 residue acidic C-terminal domain retains most RAD6-dependent functions except for sporulation (Morrison *et al.*, 1988), suggesting that there is sufficient information within the N-terminal domain of RAD6 to support most RAD6-specific *in vivo* functions.

To examine the role of the C-terminal domain of CDC34 in determining its function in cell cycle progression, we constructed several E2 variants using sequences from CDC34 and the related but functionally distinct ubiquitin conjugating enzyme RAD6. We report here that a truncation mutant of CDC34 which lacks its C-terminal domain cannot perform the essential *in vivo* function of CDC34. We also report that a construct encoding a chimeric RAD6–CDC34 ubiquitin conjugating enzyme in which the acidic C-terminal domain of RAD6 has been replaced with that of CDC34 is capable of performing the essential functions of CDC34. This chimeric E2 still supports at least some RAD6 function *in vivo*; thus, addition of the CDC34 C-terminal domain augments, but does not supplant, the *in vivo* functions of

RAD6. Moreover, only a relatively small portion of the C-terminal domain is required for CDC34 function in either CDC34 or in the chimeric construct. Congruent results have been obtained by Silver *et al.* (1992), as reported in the accompanying paper.

Results

Design and construction of variant E2s

Experimental evidence exists for a two domain structure for the class II E2 encoded by RAD6 (Morrison *et al.*, 1988). Sequence comparisons suggest that other class II E2s, including CDC34, also comprise two structural domains (Figure 1A). This assumption guided the design of our first

Fig. 1. Design of E2 constructs. **A.** Domain structure of the CDC34 and RAD6 ubiquitin conjugating enzymes. Assignment of sequence domains was based on the comparison of the amino acid sequences of CDC34 and RAD6 described in Goebel *et al.* (1988). Analysis of purified RAD6 protein verifies that it has a two domain structure, comprising a protease resistant core corresponding to the N-terminal domain attached to an extended polyacidic C-terminal domain (Morrison *et al.*, 1988). The numbers denote the amino acid residue on the N-terminal side of the sequence boundaries shown. The C-terminal domain of RAD6 consists of a 23 residue aspartate-rich region. The C-terminal extension of CDC34 comprises a 125 residue domain, embedded in which (from residues 245 to 265) is an aspartate-rich region with striking similarity to the C-terminal domain of RAD6. **B.** E2 constructs derived from CDC34 and RAD6. The designation of the E2 constructs is that used in the text. Shaded blocks represent coding sequences from CDC34; unshaded blocks represent coding sequences from RAD6. Numbering refers to the amino acid residues relative to their position in the amino acid sequences of wild-type CDC34 or RAD6. Constructs containing the N-terminal domain of CDC34 were expressed from the endogenous *CDC34* promoter (P_{CDC34}) in either a low or a high copy vector, or from the *GAL1* promoter (P_{GAL1}) in a high copy vector. Constructs containing the N-terminal domain of RAD6 were expressed from the endogenous *RAD6* promoter (P_{RAD6}) in either a low or a high copy vector, or from the *GAL1* promoter (P_{GAL1}) in a high copy vector.

Fig. 2. Complementation of a *cdc34* temperature-sensitive mutant by E2 constructs. Strain RS166 (containing the *cdc34-1* temperature-sensitive allele) transformed with vector alone (a) or vector containing *CDC34* (b), *CDC34t* (c), *CDC34-RAD6* (d), *RAD6* (e), *RAD6t* (f) or *RAD6-CDC34* (g) was streaked onto SC-Ura plates and incubated at either 23 or 37°C for several days. The vector here was either YCplac33 (CEN) or YEplac195 (2 µm).

set of E2 constructs, illustrated in Figure 1B. We constructed plasmids that contain sequences encoding either wild-type *CDC34*, a truncation variant of *CDC34* (*CDC34t*) lacking residues 171–295 (which correspond to its diverged C-terminal domain) or a chimeric E2 (*CDC34-RAD6*) made by appending sequences encoding residues 152–172 from the C-terminus of *RAD6* onto the N-terminal domain of *CDC34*. Similarly, we constructed plasmids that contain sequences encoding wild-type *RAD6*, a truncation variant of *RAD6* (*RAD6t*) lacking residues 152–172 (which correspond to the bulk of its C-terminal domain) or a chimeric E2 (*RAD6-CDC34*) made by appending sequences encoding residues 171–295 from the C-terminus of *CDC34* onto the N-terminal domain of *RAD6*. We chose the location of the deletions and chimeric junctions to correspond to where the amino acid sequences of *RAD6* and *CDC34* diverged according to the sequence alignment described by Goebel *et al.* (1988).

Sequences encoding the six E2 constructs were cloned into three vectors: the CEN (low copy) plasmid YCplac33 (Gietz and Sugino, 1988) in which expression was from the promoter originally associated with the N-terminal domain of the construct; the 2 micron (high copy) plasmid YEplac195 (Gietz and Sugino, 1988) in which expression was again from the promoter originally associated with the N-terminal domain; and a 2 micron plasmid (YEplac195-GAL, constructed from YEplac195; see Materials and methods) in which expression was from the galactose-inducible *GAL1* promoter.

Table I. Summary of complementation of *cdc34* mutants by the E2 constructs

Vector	Promoter	E2 construct	Complementation of		
			Cdc34-1	Cdc34-2	<i>cdc34::HIS3</i>
YCplac33	P_{CDC34}	Vector only	–	–	–
		<i>CDC34</i>	+++	+++	+++
		<i>CDC34t</i>	–	–	–
	P_{RAD6}	<i>CDC34-RAD6</i>	–	–	–
		<i>RAD6</i>	–	–	–
		<i>RAD6t</i>	–	–	–
YEplac195	P_{CDC34}	<i>RAD6-CDC34</i>	+	+	+
		Vector only	–	–	–
		<i>CDC34</i>	+++	+++	+++
		<i>CDC34Δ1</i>	ND	+++	+++
		<i>CDC34Δ2</i>	ND	+++	+++
		<i>CDC34t</i>	–	–	–
	P_{RAD6}	<i>CDC34-RAD6</i>	–	–	–
		<i>RAD6</i>	–	–	–
		<i>RAD6t</i>	–	–	–
		<i>RAD6-CDC34</i>	+++	+++	+++
		<i>RAD6-CDC34Δ1</i>	ND	+++	+++
		<i>RAD6-CDC34Δ2</i>	ND	+++	+++
YEplac195GAL	P_{GAL1}	Vector only	–	–	–
		<i>CDC34</i>	+++	+++	+++
		<i>CDC34t</i>	–	–	–
		<i>CDC34-RAD6</i>	–	–	–
		<i>RAD6</i>	–	–	–
		<i>RAD6t</i>	–	–	–
		<i>RAD6-CDC34</i>	+++	+++	+++

E2 constructs are described in Figures 1 and 6. Complementation of each construct was measured relative to that provided by *CDC34* (+++). A single plus (+) indicates that a construct complemented but did so more weakly than did wild-type *CDC34* (see text for explicit description of weak complementation). Complementation of *cdc34-1* and *cdc34-2* was measured by growth of the transformed ts strains at 37°C (Figure 2). Complementation of *cdc34::HIS3* was measured using the colony sectoring assay (Figures 3 and 4). ND = not determined).

Fig. 3. Colony sectoring assay for *cdc34* function. The haploid yeast strain CK125 containing a *cdc34::HIS3* disruption and an *ade2-101* ochre mutation was viably maintained by the presence of the plasmid pUN20 (Elledge and Davis, 1988) into which *CDC34* had been cloned (pUN20-CDC34, represented by the upper plasmid bearing *TRP1*, *CDC34* and *SUP11*). Because of the *ade2-101* ochre mutation, CK125 would normally yield red colonies after incubation for several days on plates that contain limiting amounts of adenine. However, since pUN20-CDC34 encodes the *SUP11* ochre suppressor, the presence of pUN20-CDC34 rendered the strains Ade⁺ and caused them to form white colonies on limited adenine plates. The pUN20-CDC34 plasmid also contained a *TRP1* marker, but this plasmid was stably maintained even in the absence of nutritional selection for *TRP1* because of the requirement for plasmid-encoded *CDC34*. To assay an E2 construct (denoted X in the lower plasmid) for *CDC34*-complementing activity, a plasmid encoding the construct was transformed into this strain and the resulting transformants struck out onto test media lacking uracil (selecting for the X-encoding plasmids which carry a *URA3* marker) but containing tryptophan and limited adenine. If X complemented *CDC34* function, then the pUN20-CDC34 was lost at a rate of several percent per generation and gave rise to white colonies containing red sectors. If X did not complement *CDC34* function, then the pUN20-CDC34 was maintained and gave rise to non-sectored colonies.

Complementation of *cdc34* temperature-sensitive mutants by variant E2s

The above constructs were transformed into two different *cdc34* ts mutant strains (RS166, containing *cdc34-1*; and MGG15, containing *cdc34-2*; see Materials and methods). Transformants were streaked out and tested for growth at permissive (23°C) and non-permissive (37°C) temperatures. Figure 2 shows the results obtained using RS166; essentially identical results were also obtained using MGG15 (Table I). The *cdc34-1* strains yielded spontaneous, temperature-resistant revertants at a very high frequency (Figure 2) whereas the *cdc34-2* strain was more stable. We tested several independent transformants for each of our constructs and obtained consistent results. The low copy and high copy

plasmids bearing wild-type *CDC34* or the RAD6–*CDC34* chimeric E2 expressed from the *CDC34* promoter complemented the temperature sensitivity of both strains (Figure 2b and g; Table I). Both low and high copy plasmids encoding RAD6, RAD6t, *CDC34*t and *CDC34*–RAD6 failed to complement either ts mutant (Figure 2c–f; Table I). This was true whether expression was from the RAD6 promoter (in the case of RAD6 and RAD6t), the *CDC34* promoter (in the case of *CDC34*t and *CDC34*–RAD6), or the *GAL1* promoter when induced on galactose-containing media (Table I).

Growth at 37°C of *cdc34*ts strains transformed with RAD6–*CDC34* expressed from the low copy plasmid YCplac33 was slower than that of the strains containing

Fig. 4. Complementation of a *cdc34* disruption strain by *CDC34* and *RAD6*–*CDC34* as determined by the colony sectoring assay for *CDC34* function. Strain CK125 containing pUN20-*CDC34* was transformed with vector alone (a and d), vector containing *CDC34* (b and e) or vector containing *RAD6*–*CDC34* (c and f), and struck out onto test media and incubated for several days. In a–c the vector was YCplac33; in d–f the vector was YEplac195.

RAD6–*CDC34* expressed from the high copy YEplac195 as judged by colony size on plates (Figure 2). Microscopic examination of *cdc34*ts strains expressing *CDC34* or *RAD6*–*CDC34* in either low or high copy plasmids and grown at 37°C confirmed that they no longer displayed the extreme multi-budded phenotype characteristic of *cdc34*-defective strains, whereas *cdc34*ts strains expressing the other variant E2s displayed the *cdc34*-defective phenotype at 37°C (data not shown).

Complementation of a *cdc34* disruption mutant by variant E2s

The above results indicated that the *CDC34* C-terminal domain was necessary for essential *CDC34* cell cycle function. Moreover, they suggested that the chimeric *RAD6*–*CDC34* E2, in which the *CDC34* C-terminal domain had been appended onto the N-terminal domain of *RAD6*, could also provide essential *CDC34* function. However, it was possible that the chimeric E2 indirectly suppressed the temperature-sensitive phenotype of the *cdc34*ts strains, e.g. by stabilizing the temperature-sensitive *cdc34* proteins through direct protein–protein interaction. Therefore, we tested whether the *RAD6*–*CDC34* chimeric E2 could complement a disruption allele of *CDC34*.

We employed a colony sectoring assay (Hieter *et al.*, 1985; Elledge and Davis, 1988) to test for *CDC34*-complementing activity (Figure 3). A plasmid encoding a construct to be tested for *CDC34*-complementing activity is transformed into

a tester strain containing a disruption of its chromosomal *CDC34*. The tester strain is viably maintained because of the presence of a *CDC34*-encoding plasmid (pUN20-*CDC34*). If the construct does not supply essential *CDC34* function, then the tester strain will maintain the pUN20-*CDC34* even in the absence of nutritional selection. If the construct does complement the *cdc34* disruption, then the pUN20-*CDC34* is lost in the absence of nutritional selection. Because pUN20-*CDC34* also carries SUP11 and because the tester strain contains an ochre mutation in *ade2*, cells that maintain pUN20-*CDC34* produce white colonies whereas cells that do not stably maintain pUN20-*CDC34* produce red sectoring colonies. Thus, the appearance of sectoring colonies indicates that the construct being tested can provide essential *CDC34* function.

When we transformed CK125 (containing pUN20-*CDC34*) with low or high copy plasmids encoding either *CDC34* or the chimeric *RAD6*–*CDC34* E2 and streaked the transformants out onto test media, we obtained red sectoring colonies (Figure 4b, c, e and f; Table I). Transformants harboring the *RAD6*–*CDC34* construct on a low copy plasmid showed weaker sectoring than those harboring *RAD6*–*CDC34* on a high copy plasmid or *CDC34* on either plasmid (cf. Figure 4c with 4b, e and f). When we restructured cells from the red or the sectoring colonies onto limited adenine plates, we obtained red colonies which were auxotrophic for tryptophan, consistent with loss of the pUN20-*CDC34* plasmid. Red colonies obtained from

transformants bearing RAD6–CDC34 on a low copy plasmid (YCplac33–RAD6–CDC34) grew more slowly than those obtained from transformants bearing either RAD6–CDC34 on a 2 micron plasmid (YEplac195–RAD6–CDC34) or wild-type CDC34 on either plasmid as judged by colony size on plates.

We isolated both YCplac33–RAD6–CDC34 and YEplac195–RAD6–CDC34 from cells forming red colonies and found by restriction analysis that these plasmids had not undergone any detectable rearrangement (data not shown). Moreover, these segregants failed to grow on 5-fluoroorotic acid (FOA)-containing media, indicating that we could not obtain viable cells lacking the URA3 (RAD6–CDC34 bearing) plasmid. This observation is consistent with the expectation that a plasmid encoding RAD6–CDC34 would be essential in the absence of pUN20–CDC34. We conclude that the sectoring phenotype of these cells was due to a bona fide shuffling of plasmids, and that the chimeric CDC34–RAD6 construct can supply the essential functions of CDC34, albeit less efficiently when present in low copy than does wild-type CDC34.

Transformation of CK125 with constructs encoding CDC34t, CDC34–RAD6, RAD6 or RAD6t did not yield sectorized colonies (Table I). Thus, E2 constructs that lack the C-terminal domain of CDC34 (including the truncation mutant of CDC34) did not complement the *cdc34::HIS3* disruption mutant.

Complementation of a *rad6* disruption mutant by variant E2s

The above results showed that the chimeric RAD6–CDC34 E2 was competent to perform all of the essential functions of wild-type CDC34. With regard to RAD6-specific activities of this chimeric E2, two possibilities existed: (i) the chimeric E2 was competent only for CDC34-specific functions and had abandoned its RAD6 functions; i.e. the presence of the C-terminal extension from CDC34 interfered with execution of RAD6-specific functions, or (ii) the chimeric E2 still retained at least some of its RAD6-specific functions; i.e. the presence of the CDC34 tail had augmented the function of the chimera while still permitting RAD6-specific functions to be carried out. To examine these possibilities, we tested the ability of the RAD6–CDC34 chimeric E2 to complement a *rad6* disruption strain with respect to its growth deficiency, sensitivity to UV and sporulation defect.

RS33-2Crad6::LEU2 and the congenic strain RS33-2C (Rad6⁺) (see Materials and methods) were transformed with the E2 constructs on both low and high-copy vectors. The RAD6, RAD6t and RAD6–CDC34 encoding constructs, on either low or high copy number plasmids, complemented the growth defect displayed by the *rad6::LEU2* strain at both 30 and 37°C as judged by growth on plates; CDC34, CDC34t and CDC34–RAD6 did not (data not shown). To test UV sensitivity, strains were plated out on SC-Ura media and irradiated with UV light at doses from 0 to 50 J/m². The fraction of survivors on these irradiated plates was then determined relative to a control (unirradiated) plate. Over the range of UV doses examined, the *rad6::LEU2* strains expressing RAD6, RAD6t and the chimeric RAD6–CDC34 from a low copy vector showed equal resistance to UV (Figure 5). Within the sensitivity of our assays, RS33-2Crad6::LEU2 strains expressing CDC34, CDC34t, CDC34–RAD6 or vector only were equally

Fig. 5. Complementation of the UV sensitivity of a *rad6* disruption strain by RAD6–CDC34. Survival after UV irradiation of RS33-2Crad6::LEU2 transformed with YCplac33 carrying no insert (■) or encoding RAD6 (●), RAD6t (▲) or RAD6–CDC34 (◆). In each case, expression of the construct was from the RAD6 promoter.

sensitive to killing by UV (data not shown). We obtained essentially the same results when we examined constructs expressed from the high copy vectors using either homologous promoters or induced GAL1 promoter (data not shown).

We also examined the ability of our constructs to complement the sporulation deficiency of a *rad6::LEU2/rad6::LEU2* diploid (Table I). The diploid *rad6* disruption strain transformed with a low copy number plasmid encoding RAD6 achieved 37% sporulation on sporulation media. A construct encoding RAD6t supported sporulation to a level of 26%. RAD6t contains two acidic residues (Glu–Asp) at its C-terminus; we surmise that this is sufficient to allow for the truncation to function in sporulation. A construct encoding the chimeric RAD6–CDC34 construct showed reduced sporulation (~7%). The diploid *rad6* disruption strain transformed with control plasmid or plasmid CDC34, CDC34t or chimeric CDC34–RAD6 showed 0% sporulation. The same relative levels of sporulation were seen when constructs were expressed from a high copy number plasmid using either their homologous promoter or the GAL1 promoter induced with galactose (data not shown). The constructs showed no effect on the efficiency of sporulation of a homozygous RAD6/RAD6 diploid, or a heterozygous *rad6::LEU2/RAD6* diploid (data not shown). We conclude that the RAD6–CDC34 chimeric E2 was still competent to perform RAD6-specific functions in sporulation, albeit with reduced efficiency.

Deletion analysis of the CDC34 C terminal domain

The ability of the RAD6–CDC34 chimeric E2 to provide the essential function of CDC34 indicates that determinants in the C-terminal domain of CDC34 exist that are sufficient to recruit the RAD6 ubiquitin conjugating enzyme for CDC34 function. To begin to address the nature and extent of these determinants, we constructed genes encoding several C-terminal deletion mutants of both CDC34 and RAD6–

Table II. Complementation of the sporulation deficiency of a diploid *rad6::LEU2/rad6::LEU2* disruption strain by E2 constructs carried on the low copy plasmid YCplac33

E2 construct	% sporulation
Vector	0
<i>RAD6</i>	37
<i>RAD6t</i>	26
<i>RAD6-CDC34</i>	7
<i>CDC34</i>	0
<i>CDC34t</i>	0
<i>CDC34-RAD6</i>	0

Fig. 6. Truncation mutants of CDC34 and RAD6-CDC34. The designation of the constructs is that used in the text. Shaded blocks represent coding sequences derived from *CDC34*; unshaded blocks represent coding sequences derived from *RAD6*. Numbering refers to the amino acid residues relative to their position in the amino acid sequences of wild-type *CDC34* or *RAD6*. *CDC34Δ1* and *CDC34Δ2* were carried in YEplac195 and expressed from the *CDC34* promoter; *RAD6-CDC34Δ1* and *RAD6-CDC34Δ2* were also carried in YEplac195, except expression was from the *RAD6* promoter. *CDC34*, *CDC34t*, *RAD6-CDC34* and *RAD6t* are the same constructs described in Figure 1 and are included here for reference.

CDC34 (Figure 6) and tested their abilities to complement the temperature sensitivity of *cdc34-2*, and the lethality of *cdc34::HIS3* as measured by the colony sectoring assay.

Deletion endpoints were selected based on 'sequence domains' suggested by the same comparison of *RAD6* and *CDC34* amino acid sequences (Goebel *et al.*, 1988; Figures 1) used to guide the construction of our previous set of E2 constructs. This comparison suggests dividing the *CDC34* C-terminal domain into three regions: one comprising residues 171–244 immediately following the N-terminal domain of *CDC34*; a region comprising residues 266–295 that resides at the C-terminal end of the C-terminal domain; and, between them, a region comprising residues 245–265 that contains a total of 16 acidic (aspartate or glutamate)

residues and that is strikingly similar to the acidic C-terminal domain of *RAD6*. We denoted as *CDC34Δ1* and *CDC34Δ2* constructs which suffered from progressively larger truncations of the *CDC34* C-terminal domain, and as *RAD6-CDC34Δ1* and *RAD6-CDC34Δ2* constructs which bore the corresponding deletions within the chimeric *RAD6-CDC34* E2 (Figure 6). All of these constructs were subcloned into YEplac195 and tested for *cdc34*-complementing activity within that vector.

The deletion mutants *CDC34Δ1* (which lacked sequences encoding residues 266–295) and *CDC34Δ2* (which lacked sequences encoding residues 245–295) complemented the temperature sensitivity of *cdc34-2* and complemented the lethality of *cdc34::HIS3* as measured by the colony sectoring assay (Table I). The corresponding chimeric deletion mutants *RAD6-CDC34Δ1* and *RAD6-CDC34Δ2* also complemented both strains (Table I). Thus, the region comprising residues 171–244 was sufficient for *CDC34* function when appended to the N-terminal domain of *RAD6*. We conclude that the C-terminal determinant of essential *CDC34* function lies within residues 171 to 244 in the C-terminal domain.

Discussion

Previous studies have shown that deletion of the C-terminal domain of *CDC34* abolishes its ability to ubiquitinate histones *in vitro* (Goebel *et al.*, 1988). We have shown here that deletion of the C-terminal domain of *CDC34* abolishes its essential cell cycle function *in vivo*. Furthermore, we have extended these results by demonstrating a positive role for the C-terminal domain in *CDC34* function: namely, its ability to recruit an apparently functionally distinct E2 (*RAD6*) for *CDC34* function. Silver *et al.* (1992) report congruent results in the accompanying paper. A likely interpretation of these results assumes that the functional distinction between *RAD6* and *CDC34* reflects a difference in their substrate specificity *in vivo*. In this view, addition of the C-terminal domain of *CDC34* to *RAD6* recruits *RAD6* for *CDC34* function by targeting this chimeric E2 to *CDC34*-specific substrates whose ubiquitination is necessary for the *G*₁ to *S* transition of the cell cycle. Such targeting could be due to direct interaction between the *CDC34* C-terminal domain and *CDC34*-specific substrates as originally proposed by Goebel *et al.* (1988) and Jentsch *et al.* (1990). An analogous effect of the acidic C-terminal extension of the *Arabidopsis thaliana* UBC4 *in vitro* has been reported (Sullivan and Vierstra, 1991): deletion of the C-terminal domain of *A.thaliana* UBC4 eliminates efficient ubiquitin conjugation to histones *in vitro*, whereas fusion of the UBC4 C-terminal domain to UBC1 (which is normally incapable of efficient ubiquitin conjugation to histones) results in a chimeric protein capable of histone conjugation. Kinetic measurements suggest that the specificity of the UBC4 and the UBC1-UBC4 chimera is due to direct, electrostatic interactions between the UBC4 C-terminal domain and histones (Sullivan and Vierstra, 1991).

Alternatively, the C-terminal domain of *CDC34* could govern substrate selection indirectly, through interaction with *trans*-acting factors required for substrate selection by *CDC34*, such as a putative *CDC34*-specific ubiquitin protein ligases. Although previous speculation suggested that the

C-terminal extensions of class II E2s supplied E3-like functions (Jentsch *et al.*, 1990), it now seems likely that the class II E2s operate *in vivo* in conjunction with one or more specific and as yet unidentified E3s. Indeed, the class II E2 encoded by RAD6 operates in the N-end rule pathway in conjunction with an E3 encoded by the yeast gene UBR1 (Dohmen *et al.*, 1991) while another putative RAD6-specific E3 activity distinct from that encoded by UBR1 has been identified biochemically (Sharon *et al.*, 1991).

A substantial portion of the C-terminal domain of CDC34 (residues 245–295) is dispensable for essential CDC34 function. Moreover, the remaining portion of the CDC34 C-terminal domain (comprising residues 171–244) is not only sufficient for CDC34 function, but is also sufficient to recruit the N-terminal domain of RAD6 for CDC34 function when appended to its C-terminus. We speculate that the apparently non-essential portion of the CDC34 C-terminal domain plays a non-essential, but facilitating or regulatory role in essential CDC34 function. The CDC34 C-terminal domain may also be required for other CDC34-specific functions that are not essential for vegetative growth or that are also met by other, redundant ubiquitin conjugating activities in yeast. Redundancy among yeast ubiquitin conjugating enzymes has been noted previously: the yeast E2s encoded by UBC1, UBC4 and UBC5 appear to have overlapping functions since single mutations in each of them results in relatively modest phenotypes whereas a triple mutation that inactivates all three is lethal (Seufert *et al.*, 1990).

While CDC34 and RAD6 both catalyze multi-ubiquitination *in vitro*, the lysine residue on ubiquitin used for ubiquitin–ubiquitin conjugate formation apparently differs between chains formed by CDC34 and RAD6 (Haas *et al.*, 1991). Specifically, a ubiquitin mutant in which Lys48 has been replaced by arginine blocks CDC34-catalyzed multi-ubiquitin chain formation *in vitro*. This result shows that the ubiquitin–ubiquitin linkages formed by CDC34 are constrained to be between the C-terminus of ubiquitin and the ϵ -amino group of Lys48 on ubiquitin. In contrast, this same mutant ubiquitin supports RAD6-catalyzed multi-ubiquitin chain formation, indicating that RAD6 is not restricted to multi-ubiquitin chain formation via Lys48 (Haas *et al.*, 1991). It is possible that differences in lysine selection in multi-ubiquitin chain formation by CDC34 and RAD6 are responsible in part for the functional distinction between the two ubiquitin conjugating enzymes *in vivo*; however, our results begin to place constraints on such a view. Thus, if the Lys48 linkage in the ubiquitin–ubiquitin chain formed by CDC34 is required for CDC34-dependent function, then the ability to substitute RAD6–CDC34 for CDC34 *in vivo* implies that either the N-terminal domain of RAD6 can synthesize Lys48-linked multi-ubiquitin chains, or that the addition of the CDC34 tail ‘reprograms’ the selection of the lysine residue used for multi-ubiquitination by RAD6, possibly via involvement of a CDC34-specific ubiquitin protein ligase. If the lysine used for multi-ubiquitination by the RAD6–CDC34 chimeric E2 *in vivo* differs from that of CDC34, then formation of a Lys48-linked multi-ubiquitin chain is not required for the expression of CDC34 function.

An obvious question is whether the C-terminal domain of CDC34 can confer CDC34 function onto other yeast E2s when added to their C-termini. We have constructed other chimeric E2s in which the C-terminal domain of CDC34

has been appended onto either UBC4 (a class I E2 which lacks a C-terminal extension) or the N-terminal domain of UBC1 (another class II E2), but, thus far, we have been unable to complement any *cdc34* mutation with these chimeric E2s (unpublished data). It remains for us to rule out trivial reasons (e.g. misfolding or improper intracellular localization) for the failure of these constructs to complement *cdc34* mutations; however, given the clear functional specificity of the class I E2 enzymes that lack C-terminal extensions (Jentsch *et al.*, 1991), it does not seem implausible that determinants in the N-terminal domain of both CDC34 and RAD6–CDC34 are also required for essential CDC34-dependent functions. A *rad6* deletion mutant shows an apparent cell cycle arrest at elevated temperature (Ellison *et al.*, 1991), suggesting that RAD6 may play a normally cryptic role in cell cycle progression; we speculate that the N-terminal domains of CDC34 and RAD6 may interact with some of the same factors to execute functions involved in cell cycle progression. Predisposal of the RAD6 N-terminal domain towards interaction with these factors may account for the ability of the RAD6–CDC34 chimeric E2 to function in CDC34-dependent pathways when other chimeric E2s appear not to function in CDC34-dependent pathways; other chimeric E2s containing the C-terminal domain of CDC34 would lack CDC34-function because they lack the ability to interact with such factors via their N-terminal domains.

Although fusion of the CDC34 C-terminal domain to the RAD6 N-terminal domain recruits the RAD6 ubiquitin conjugating enzyme for function in CDC34-dependent pathways, the chimeric E2 still acts in at least some RAD6-dependent pathways. Mutations in RAD6 are pleiotropic with phenotypes including sensitivity to a variety of DNA-damaging agents, deficiency in sporulation, deficiency in induced mutagenesis and stimulation of Ty transposition (Prakash *et al.*, 1990). Also, the ubiquitin conjugating activity of RAD6 is responsible for the ubiquitination of proteolytic substrates of the N-end rule pathway (Dohmen *et al.*, 1991; Sung *et al.*, 1991), though none of the other phenotypes of *rad6* mutations have been ascribed to a defect in the N-end rule pathway. The chimeric RAD6–CDC34 E2 complements the growth deficiency, UV sensitivity and (to a lesser extent) the sporulation deficiency of a *rad6* disruption mutant. The complementation of at least some RAD6-dependent functions by the chimeric RAD6–CDC34 E2 indicates that this construct is able to engage in those interactions necessary for its function in these pathways. This raises the possibility that other factors involved specifically in either RAD6- or CDC34-dependent ubiquitination pathways might be identified based on ‘competition’ between different pathways for limiting amounts of the chimeric RAD6–CDC34 enzyme. For example, overexpression of a RAD6-interacting protein in a yeast strain in which CDC34 function is being supplied by the RAD6–CDC34 chimeric E2 may result in a G₁/S arrest, if interaction of RAD6–CDC34 with an excess of this factor precludes interaction of RAD6–CDC34 with CDC34 pathway-specific factors. We also predict that it should be possible to identify mutations in yeast that yield a ‘synthetic phenotype’ such that the mutant yeast is viable in the presence of CDC34, but is unable to substitute the RAD6–CDC34 chimeric E2 for CDC34 (or vice versa). We expect such mutations to arise in genes whose products work intimately with either CDC34 or RAD6 *in vivo*.

Materials and methods

Construction of CDC34, RAD6 and chimeric E2 expression plasmids

Recombinant DNA manipulations and verification of constructs by restriction analysis and double-stranded sequencing was performed using standard procedures. Details regarding the precise conditions used for polymerase chain reactions are available on request. A 1.2 kb fragment encoding CDC34 with its native promoter was obtained by polymerase chain reaction (PCR) amplification of pGEMB/S34 plasmid DNA with the primers CDC34-5' (GGGTCGACAAAGAGCAATAAGAG) and CDC34-3' (GGGCAT-GCCTTCTCCCTGTCAATC). A 1.4 kb fragment encoding RAD6 with its native promoter was obtained by amplification of genomic DNA from strain BWG1-7a (*MATa his5-519 ura3-52 ade1-100 leu2-3,112*) with the primers RAD6-5' (GGGTCGACAAAGCTTGGGATTGGGTTA) and RAD6-3' (GGGCATGCAAACTGGGGTCACTCTC). The 5' and 3' primers were designed to encode *Sall* and *SphI* restriction sites at their ends, respectively, for use in subsequent cloning. The amplified fragments were cloned between the *Sall* and *SphI* sites into both YCplac33 and YEplac195. The amplified fragment encoding CDC34 was also cloned between the *Sall* and *SphI* sites into the plasmid pUN20 (Elledge and Davis, 1988) to create pUN20-CDC34.

Fragments that encoded chimeric E2s were constructed by recombinant PCR (Higuchi, 1989). Briefly, to obtain a fragment encoding the chimeric CDC34–RAD6 E2, a 0.7 kb fragment encoding the N-terminal 170 residues of CDC34 was first obtained by amplifying pGEMB/S34 DNA with the primers CDC34-5' and CDC34/RAD6-3' (CATATCGTCCATATCATC-TTGTTTCGATCTTTC), while a 0.5 kb fragment encoding the C-terminal 21 residues of RAD6 was obtained by amplifying genomic DNA with the primers CDC34/RAD6-5' (AGATCGAAACAAGATGATATGGACGAT-ATGGAC) and RAD6-3'. The two fragments were purified, mixed and used as template for an amplification using the primers CDC34-5' and RAD6-3'. The product of this final amplification reaction was a 1.2 kb fragment encoding the CDC34–RAD6 chimeric E2. Similarly, to obtain a fragment encoding the chimeric RAD6–CDC34 E2, a 0.9 kb fragment encoding the N-terminal 151 residues of RAD6 was obtained by amplifying genomic DNA with the primers RAD6-5' and RAD6/CDC34-3' (GAAACCTTTAGGGATATCCTCCCAAGATTTCTC), while a 0.4 kb fragment encoding the C-terminal 125 residues of CDC34 was obtained by amplifying pGEMB/S34 DNA with the primers RAD6/CDC34-5' (AAATCTTGGGAGGATATCCCTAAAGTTTCATA) and CDC34-3'. These two fragments were purified, mixed and used as template for a final amplification using the primers RAD6-5' and CDC34-3', yielding a 1.4 kb fragment encoding the RAD6–CDC34 chimeric E2. As above, the fragments encoding the chimeric E2s were cloned between the *Sall* and *SphI* restriction sites in YCplac33 and YEplac195.

A truncation mutant of CDC34 (CDC34t) which lacked the 125 residue C-terminal domain was obtained by amplification of pGEMB/S34 DNA using the primers CDC34-5' and CDC34t-3' (GGGGGGGCGATGCTG-CAGTCAATCTTGTTCGATCT). A truncation mutant of RAD6 (RAD6t) which lacked the acidic 21 residue C-terminal domain was obtained by amplification of genomic DNA using the primers RAD6-5' and RAD6t-3' (GGGGGGGCGATGCTGCAATCCTCCCAAGATT). Fragments encoding RAD6t or CDC34t were cloned between the *Sall* and *SphI* sites in YCplac33 and YEplac195. Fragments encoding other truncation mutants of CDC34 which lacked 30 residues (CDC34Δ1) or 51 residues (CDC34Δ2) from their C-termini were obtained by amplification of pGEMB/S34 DNA using the primers CDC34-5' and either CDC34Δ1-3' (GGGGGCGATGCTT-AATCTATACTATCGTCGTC) or CDC34Δ2-3' (GGGGGCGATGCTT-AAGTTGCTGCTTATAGTTGTA) respectively. Similarly, fragments encoding the corresponding truncation mutants of RAD6–CDC34 (RAD6–CDC34Δ1 and RAD6–CDC34Δ2) were obtained by amplification of YEplac195RAD6–CDC34 with primers RAD6-5' and either CDC34Δ1-3' or CDC34Δ2-3'. These truncation mutants were subcloned between the *Sall* and *SphI* sites of YEplac195.

To place constructs encoding various E2s under GAL control, we first isolated from plasmid pJDPS1 (obtained from J.Dohmen) a 0.6 kb *EcoRI*–*BamHI* fragment containing the GAL1/GAL10 promoter region and subcloned it between the *EcoRI* and *BamHI* restriction sites of YEplac195 to form YEplac195GAL. We obtained fragments encoding CDC34, RAD6, CDC34t, RAD6t, CDC34–RAD6 and RAD6–CDC34, but lacking their native promoters, by amplifying template DNA with an appropriate 3'-primer and either CDC34-2-5' (GGGTCGACATATGAGTAGTCGCA) or RAD6-2-5' (GGGTCGACATATGTCACACACAGCT). These fragments were then cloned between the *Sall* and *SphI* sites of YEplac195GAL.

Yeast strains

MGG15 (*MATa his3-Δ1 ura3-52 cdc34-2*) and a heterozygous *cdc34* disruption strain (*MATa/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 cdc34::HIS3/CDC34*) were obtained from M.Goebl. CK125 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 cdc34::HIS3*) transformed with pUN20-CDC34 was obtained by sporulating and germinating the heterozygous *cdc34/CDC34* diploid transformed with pUN20-CDC34. RS166 (*MATa his1-1 trp2 ura3-52 leu2-3,112 cdc34-1*) transformed with pRS112 (derived from YE24 by insertion into its *BamHI* site of a *Sau3A* partial insert containing *CDC34*), RS33-2C (*MATa leu2-3,112 ura3-52 trp1-289 arg4-17 his4-17*), Y433 (*MATα ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ98*), and the congenic *rad6* disruption strains RS33-2Crad6::LEU2 and Y433rad6::LEU2 were obtained from R.Shiestl (Schiestl and Prakash, 1989). Because we observed that the mutant strain RS166 yielded temperature-resistant phenotypic revertants with high frequency (see e.g. Figure 2, where a high frequency of phenotypic reversion is seen in several of the plate sectors), we stored and propagated RS166 while transformed with the *CDC34*-complementing plasmid pRS112. The pRS112 plasmid was evicted from RS166 by growth on 5-FOA. The resulting Ura[−] temperature-sensitive yeast was immediately used in these experiments. MGG15 yielded temperature-resistant phenotypic revertants at a much lower frequency.

Yeast genetics and assays

Yeasts were propagated on synthetic complete (SC) media with appropriate selective omissions as described by Sherman *et al.* (1986). Lithium acetate transformation of yeast was performed by the method of Schiestl and Gietz (1989). Plasmids were recovered from transformed yeast by electroporation into *Escherichia coli* following glass bead lysis and phenol–chloroform extraction of the lysis mixture (Strathern and Higgins, 1991). Plasmid sectoring shuffle assays that tested the ability of strains to propagate after the loss of pUN20-CDC34 were performed essentially as described by Hieter *et al.* (1985) and Elledge and Davis (1988), except that a concentration of 18 μg/ml of adenine hemisulfate was used to facilitate the formation of red colonies. Yeast strains being assayed for a sectoring phenotype were streaked onto test media and incubated at either 23 or 30°C for 1 to 2 days, followed by incubation at 23°C for several days to permit optimal formation of color. We saw no difference in behavior among any of our constructs between these two incubation protocols. Yeast transformants containing constructs expressed from *GAL1* were first streaked onto galactose-containing media, grown to colonies for several days, and then streaked again onto the appropriate media containing galactose to assay for function of the constructs. Diploid *RAD6/RAD6*, *rad6::LEU2/RAD6* and *rad::LEU2/rad6::LEU2* yeasts were formed by mating Y433 with RS33-2C, Y433 with RS33-2Crad6::LEU2 and Y433rad6::LEU2 with RS33-2Crad::LEU2 respectively. Sporulation of diploids was induced by patching cells onto McCleary's solid media or McCleary's solid media containing galactose in place of glucose for assay of *GAL1* promoter-driven constructs. Sporulation was measured as the percentage of asci formed after 5 days on McCleary's solid media at 30°C, counting at least 500 cells. UV irradiation of yeast was performed with a Stratagene Stratalinker UV Crosslinker (using 254 nm UV bulbs). The measurement and delivery of UV dosages was governed by the internal sensor. Survival was measured as a fraction of colonies formed on an irradiated plate relative to the number of colonies formed on an unirradiated control plate spread with an equal amount of culture.

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