A Site-directed Approach for Constructing Temperature-sensitive Ubiquitin-conjugating Enzymes Reveals a Cell Cycle Function and Growth Function for RAD6*

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We have determined the gene sequence of a temperature-sensitive allele of the cell cycle-related ubiquitin-conjugating enzyme CDC34 (UBC 3) from Saccharomyces cerevisiae. The basis of temperature sensitivity is a missense mutation resulting in a proline to serine substitution at a residue that is conserved in all ubiquitin-conjugating enzymes identified thus far. This observation raised the possibility that other temperature-sensitive ubiquitin-conjugating enzymes could be generated in the same way. We therefore created the corresponding substitution in the DNA repair-related ubiquitin-conjugating enzyme, RAD6 (UBC2), and examined the effect of temperature on the cell proliferation and DNA repair-related functions of this altered polypeptide. Yeast strains carrying this mutation proved to be temperature-sensitive with respect to cell proliferation but not with respect to the DNA damage-processing phenotypes exhibited by other rad6 mutants. Upon further investigation of the proliferation defect exhibited by this mutant, we discovered that other rad6 gene mutants deleted for the gene undergo cell cycle arrest at the nonpermissive temperature, whereas the engineered temperature-sensitive allele showed no evidence of a cell cycle defect. From these findings, we conclude that the proliferation function of RAD6 can be subdivided into a growth component and a cell division cycle component and that the growth component is unrelated to the DNA repair functions of RAD6. A reasonable interpretation of these results is that different proteins are targeted for ubiquitination in each case. The conserved proline residue of RAD6 and CDC34 is part of a turn motif common to all ubiquitin-conjugating enzymes. It is therefore likely that site-directed substitution of prolines located in turns can be generally applied for the creation of other temperature-sensitive ubiquitin-conjugating enzymes and possibly other proteins as well.

The selective transfer of ubiquitin to other proteins is an important feature of cell cycle control (1, 2), oncogenesis (3, 4), DNA repair (5), mutagenesis (5), and the stress response (6, 7). The attachment of ubiquitin to targeted substrates is mediated by the ubiquitin-conjugating enzymes (E2s) (8). Although all the E2s characterized to date are structurally related, they fall into several functionally distinct categories. The yeast CDC34 (UBC3) E2, for example, is required for the G1 to S phase transition of the cell cycle (1), whereas the yeast RAD6 (UBC2) E2 is involved in a variety of processes, including DNA repair, mutagenesis, and cell proliferation (9). In the case of RAD6, it is not clear whether or not each of the above functions represent distinct biochemical processes, or rather, are different aspects of the same process.

The utility of conditional lethal mutations in genetic and biochemical studies has long been recognized in the determination of protein structure/function relationships. In the present work, we have identified the basis of temperature sensitivity for a conditional lethal allele of CDC34 and have applied this information to create a temperature sensitive derivative of RAD6.

The phenotypic effects of this and other mutations indicate that RAD6 plays a role in both cell growth and cell division, two distinct aspects of cell proliferation, and that at least one of these two functions bears no relationship to the DNA repair-related functions of RAD6.

MATERIALS AND METHODS

Cloning and Characterization of cdc34-1—Genomic DNA from a Saccharomyces cerevisiae strain (mGG16) containing the cdc34-1 gene (genotype, Mat a, cdc34-1, ade2, leu2, arg1; a gift from K. Madura, Massachusetts Institute of Technology) was used as the template for the polymerase chain reaction with primers hybridizing to the 5' and 3' ends of the CDC34 gene (N-terminal primer, 5'-GCA TCTTGCACCGGACTACGTG-3'; C-terminal primer, 5'-GCTCTTGTGGTGGTTAACGCTTTCTC-3'). Included in the sequences for each primer were the restriction sites SacI and KpnI located in the plasmid polylinker. Twelve independent clones of KSM13-cdc34-1 were isolated and pooled. Three N-terminal coding sequence deletions of the KSM13-cdc34-1 pool were created by deleting the fragments between the SacI site and each of the sites BamHI, EcoRI, or EcorV contained within the cdc34-1 coding region. Similarly, three C-terminal coding sequence deletions were created by deleting fragments between the KpnI site and the BamHI, EcoRI, or EcorV sites. The coding strand of each of these plasmid deletions was sequenced using the M13 universal primer proximal to the SacI site of KSM13+. The complementary strand was sequenced using the M13 reverse universal primer next to the KpnI site.

RAD6 Expression Vector Construction—A RAD6 gene cassette was
amplified by the polymerase chain reaction from the cloned RAD6 gene on plasmid pRK223-3 (4) using an N-terminal primer that positioned an EcoRI site upstream of Met' (5"GCTAGAATTCA-
TAGTACCTACGAGAAGG-3") and a C-terminal primer that positioned a KpnI site downstream of the stop codon (5'-
GCTAGGTGCTTCCAGTCTGCCTTGCG-3'). The amplified gene was then cloned into the pBSM13 vector (Stratagene Inc.) between the EcoRI and KpnI sites of the plasmid polylinker to facilitate further manipulation. The codon for Pro64 is situated between the engineered EcoRI site and a nearby XmnI site. It was therefore possible to amplify an EcoRI-XmnI fragment that incorporated a Pro64 to Ser64 codon substitution using the N-terminal primer described above and a new primer that flanked both the XmnI site and position 64 (5'-GGATGAAACATTTCACTCAAAAATTTGACAT-
GCGGTTGCTTTTTGATATTT-3'). rad6 S64 was then created by replacing the EcoRI-XmnI fragment of pBSM13-RAD6 with its Ser64 counterpart. The presence of the mutation was confirmed by sequencing the entire cassette. Each cassette was then positioned between the yeast CUP1 promoter and the yeast CYC1 transcriptional terminator of the yeast CUP1 indicator. These elements were used to place a copy of the rad6 S64 gene under control of the yeast CYC1 gene promoter. 

RESULTS

Strains of S. cerevisiae that are homozygous for the temperature-sensitive cdc34-1 allele of the ubiquitin-conjugating enzyme CDC34 exhibit total cell cycle arrest in G1, resulting in cell death at the nonpermissive temperature (1). To determine the nature of the mutation conferring temperature sensitivity on the cdc34-1 protein, we sequenced the gene and found a single G to T transition that changed the proline codon GCC at position 71 to the serine codon TCC. A computer-assisted analysis of the published protein sequences of the eight known E2s from yeast (1, 5, 7, 12) and higher organisms (13-15) (Fig. 1) indicated that Pro71 of CDC34 is one of 16 amino acids that are conserved throughout all of these proteins. In all cases, this proline is situated 24-25 residues from the active site cysteine of each E2 and is found as the first proline in a Pro-X-X-Pro-Pro motif that is conserved in all but one E2.

The conservation of Pro71 raises the possibility that the Pro-X-X-Pro-Pro motif forms a common structural feature of the E2s that is required for thermal stability of these proteins. We tested this possibility by mutating the corresponding proline (Pro54) of the RAD6 E2 to serine. RAD6 was selected because of the clearly discernible phenotypes exhibited by rad6 mutants, including reduced rates of cell proliferation, increased cell killing by ultraviolet (UV) light, and a lack of mutation induced by DNA-damaging agents (9).

Effect of Ser64 on Cell Proliferation—Plasmids expressing either the wild type gene (RAD6), the Pro64 to Ser64 mutation (rad6 S64) or no gene (rad6Δ) were introduced into a yeast strain deleted for the endogenous RAD6 gene and then tested for proliferation at the permissive (30 °C) or nonpermissive (39 °C) temperature (Fig. 2). At 30 °C, the proliferation characteristics of RAD6 and rad6 S64 strains were indistinguishable, based on colony size. The colonies formed from the rad6Δ strain were markedly smaller, consistent with the proliferation defect previously reported for rad6 mutants. At 39 °C, however, both rad6Δ and rad6 S64 mutants showed a strong proliferation deficit in comparison with the RAD6 strain. rad6Δ cells failed to proliferate at the elevated temperature and lost viability with time. Neither of these rad6 phenotypes have been previously reported. rad6 S64 cells on the other hand proliferated extremely slowly with only slight loss in viability over time.

Phenotypes involving reduced proliferation can arise from either metabolic growth defects or from defects related to specific stages of the cell division cycle (cdc). cdc temperature-sensitive mutants can be readily distinguished from other types of growth temperature-sensitive mutants by a change in the proportion of budded cells in asynchronous cultures of yeast that have been shifted to the nonpermissive temperature (18). Daughter cell bud formation begins at the end of the G1 phase of growth.
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phase and progresses through the cell cycle until daughter cell separation occurs following mitosis. cdc temperature-sensitive mutants that arrest prior to bud formation will therefore show a reduction in budded cells with time following the temperature shift, whereascdc temperature-sensitive mutants that arrest subsequent to bud formation will show an increase in the proportion of budded cells.

In order to determine if the observed temperature-sensitive proliferation defect of the rad6 S64 mutant was due to a cdc defect, we examined the relative proportion of budded cells as a function of time in RAD6, rad6Δ, and rad6 S64 strains grown at 30 °C throughout or shifted to 39 °C (Fig. 3). At the permissive temperature, the percentages of budded cells observed for all three strains were indistinguishable from one another, ranging between 60–65%. The fact that the rad6Δ strain behaved similarly to the RAD6 strain under these conditions indicated that, at 30 °C, the growth defect observed for rad6Δ was not related to the cell cycle in an obvious manner (see “Discussion”).

Upon a shift to the nonpermissive temperature, all three strains showed the initial reduction in the percentage of budded cells characteristic of the transient G1 arrest that is known to occur after heat shock (19). Although the levels of budded cells observed for RAD6 and rad6 S64 strains returned close to the values observed at 30 °C, rad6Δ showed the marked increase in budded cells expected of a cdc defect occurring subsequent to bud formation. At later times following the temperature shift, a preponderance of rad6Δ cells displayed the dumbbell morphology associated with cdc defects in late S or G2 (18). The fact that the rad6 S64 mutant did not display the marked arrest phenotype of rad6Δ, yet had a significant growth defect at elevated temperature, indicated that the RAD6 proliferation function had two components: one involved in cell division and the other involved in cell growth.

The S64 Substitution Does Not Affect the DNA Repair-related Functions of RAD6—rad6 mutants are extremely sensitive to UV light and display suppressed levels of UV-induced mutagenesis relative to their wild type counterparts (9). The effects of the Ser64 substitution on UV survival and UV mutagenesis are shown in Fig. 4 and Table I, respectively. In direct contrast to the proliferation results, the UV survival curves of the rad6 S64 strain and the wild type RAD6 strain were indistinguishable from one another at both the permissive and the nonpermissive temperatures. Similarly, when the levels of UV-induced mutagenesis in these two strains were compared by measuring the reversion frequency of the ade2-101 allele, the rad6 S64 mutant failed to show the characteristic immutable phenotype of rad6 deletion mutants at either temperature. Taken together these results indicate that the DNA repair-related functions of rad6 are unaffected by the S64 substitution.

DISCUSSION

Cell growth and division are necessary components of cell proliferation. From the differential effects observed here for
the rad6Δ and rad6 S64 mutants, we conclude that RAD6 plays an important role in both cell growth and cell division. Haladus et al. (20) have previously observed an uncharacteristic and complex effect of the rad6-1 mutant on the cell cycle at 30 °C in which both G1 and S phases are lengthened at the expense of the G2 and M phases, without any effect on the budding cell ratio (20). Although the results of these workers and our own are not directly comparable, they are not inconsistent with the proliferation defect observed for the rad6Δ mutant at 30 °C. We suggest therefore that the complex phenotype observed by these authors results from the superimposition of a growth defect and a partial cell cycle defect at the permissive temperature, with the latter becoming more severe at elevated temperatures.

The reason for the temperature dependence of the rad6Δ cdc arrest phenotype observed here is unclear. We speculate that another ubiquitin-conjugating enzyme partially complements for a cell cycle function of RAD6 at 30 °C but fails to do so at the elevated temperature. This possibility is currently under investigation.

In addition to CDC34, RAD6 represents the second example of a ubiquitin-conjugating enzyme that strongly influences progression of the cell cycle. Recently Giotzer et al. (2) have reported that a prion phenotype results from the conserved motif Pro-X-X-Pro-Pro that is found in all ubiquitin-conjugating enzymes identified so far. Closely spaced proline residues are incompatible with secondary structure in proteins and are commonly located in reverse turns that link regions of secondary structure (23). We hypothesize therefore that the conserved Pro-X-X-Pro-Pro motif forms a rigid turn in E2s that is critical for maintaining the spatial relationship between two regions of secondary structure required for some aspect of target recognition. In several ubiquitin-related processes, the correct targeting of ubiquitin to proteins is known to depend on the obligatory attachment or release of ubiquitin from the active site cysteine (24, 25); therefore, the Pro-X-Pro-Pro motif may be an important determinant in the stabilization of the E2-E3 interaction.

The fact that both RAD6 and CDC34 can be made temperature-sensitive by substitution of an amino acid common to all E2s leads us to believe that other temperature-sensitive E2s can be engineered from a variety of organisms using the same general strategy. One potential application of conditional E2 mutants would be in the isolation of mutants and, ultimately, genes that suppress the temperature-sensitive phenotype, i.e. the so-called extragenic suppressors. Gene candidates for such suppressors would include mutations in E3 proteins and possibly the protein targets of E3 action.

Finally, we suggest that the destabilization of protein turns by the specific replacement of proline residues confined to the turn may represent a general strategy for the construction of temperature-sensitive proteins of diverse functions.

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REFERENCES
1. Goebel, M. G., Yochem, J., Jentsch, S., McGrath, J. P., Vars-

havsky, A., and Byers, B. (1988) Science 241, 1331–1335
2. Giotzer, M., Murray, A. W., and Kirschner, M. W. (1991) Nature 349, 132–138
3. Scheffner, M., Werness, B. A., Hubregtse, J. M., Levine, A. J., and Howley, P. M. (1989) Cell 63, 1129–1136
4. Ciechanover, A., DiGiuseppe, J. A., Berezovs, B., Orian, A., Richter, J. D., Schwartz, A. L., and Bordeur, G. M. (1991) Proc

TABLE I
rad6 S64 and RAD6 type strains show similar levels of UV-induced mutagenesis at elevated temperature

| Strain       | Temperature | Mutation frequency |
|--------------|-------------|--------------------|
|              | 30°C        | <9                 |
| RAD6         | 30          | 3400 (77)          |
| rad6 S64     | <14         | 300 (58)           |
|              | 39          | 6200 (100)         |
|              | <14         | 100 (30)           |

The reversion frequency of the ade2-101 ochre allele for yeast strains expressing RAD6 or rad6 S64 was determined as follows. Cells were grown to mid-exponential phase and spread on minimal media (Fig. 2) agar plates lacking adenine. Plates were either exposed to a 254 nm UV dose of 40 J/m² or left unirradiated. Duplicate plates were incubated at 30 or 39 °C until Ade revertant colonies were visible. Cell survival was measured in the same manner, except that plates contained adenine. Mutation frequency refers to the number of mutants detected per 10⁶ survivors. The percentage of surviving cells following UV irradiation (parenthesized values) was determined from the number of colonies formed following UV treatment divided by the number of colonies formed without UV treatment.

at the nonpermissive temperature but is inadequate for growth. In normal yeast cells, however, levels of RAD6 mRNA increase rapidly (4–8-fold) after exposure to UV light but remain unchanged in response to elevated temperatures (21). These findings imply that cells actually require more of the RAD6 protein to overcome the deleterious effects of UV light than for growth or cell division, which is in direct contradiction to the explanation provided above.

An alternative explanation that we favor is that S64 exerts its effect directly on a region of the RAD6 polypeptide responsible for the growth function with little or no effect on other functions. This defect is not likely to involve either the attachment or release of ubiquitin from the active site cysteine since defects in these steps adversely affect all RAD6 functions (22). The simplest explanation for the separable rad6 phenotypes therefore is that different proteins are ubiquitinated in each of these processes and that high temperature interferes with the targeting of ubiquitin by rad6 S64 to the specific protein substrate(s) required for growth but not those involved in cell division or DNA damage processing. Alternatively, other E2s could partially complement the rad6 S64 defect by ubiquitinating the protein(s) involved in the response to UV or cell division at high temperature but not those required for growth.

Pro64 of RAD6 and Pro34 of CDC34 are the first polypeptides in the conserved motif Pro-X-X-Pro-Pro that is found in all ubiquitin-conjugating enzymes identified so far. Closely spaced proline residues are incompatible with secondary structure in proteins and are commonly located in reverse turns that link regions of secondary structure (23). We hypothesize therefore that the conserved Pro-X-X-Pro-Pro motif forms a rigid turn in E2s that is critical for maintaining the spatial relationship between two regions of secondary structure required for some aspect of target recognition. In several ubiquitin-related processes, the correct targeting of ubiquitin to proteins is known to depend on the obligatory association of E2s with a class of proteins known as ubiquitin protein ligases or E3s (24, 25); therefore, the Pro-X-Pro-Pro motif may be an important determinant in the stabilization of the E2-E3 interaction.
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5. Jentsch, S., McGrath, J. P., and Varshavsky, A. (1987) Nature 329, 131–134
6. Finley, D., Özbuyuk, E., and Varshavsky, A. (1987) Cell 48, 1035–1046
7. Seufert, W., and Jentsch, S. (1990) EMBO J. 9, 543–550
8. Jentsch, S., Seufert, W., Sommer, T., and Reins, H.-A. (1990) Trends Biochem. Sci. 15, 195–198
9. Haynes, R. H., and Kunz, B. A. (1981) in The Molecular Biology of the Yeast Saccharomyces cerevisiae: Life Cycle and Inheritance (Strathern, J., Jones, E., and Broach, J., eds.) pp. 371–414, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Ecker, D. J., Khan, M. I., Marsh, J., Butt, T. R., and Croake, S. T. (1987) J. Biol. Chem. 262, 3524–3527
11. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
12. Seufert, W., and Jentsch, S. (1990) EMBO J. 9, 543–550
13. Reynolds, P., Koken, M. M., Hoeijmakers, J. H. J., Prakash, S., and Prakash, L. (1990) EMBO J. 9, 1423–1430
14. Schneider, R., Eckerskorn, C., Lottspeich, F., and Schweiger, M. (1990) EMBO J. 9, 1431–1435
15. Sullivan, M. L., and Vierstra, R. D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9861–9865
16. Devereux, J., Haeberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395
17. Seufert, W., McGrath, J. P., and Jentsch, S. (1990) EMBO J. 9, 4535–4541
18. Hartwell, L. H. (1974) Bacteriol. Rev. 38, 164–198
19. Johnston, G. C., and Singer, R. A. (1980) Mol. & Gen. Genet. 178, 357–380
20. Haladuc, E., Swietlinska, Z., Zaborowska, D., and Zik, J. (1982) J. Bacteriol. 152, 517–520
21. Madura, K., Prakash, S., and Prakash, L. (1990) Nucleic Acids Res. 18, 771–778
22. Sung, P., Prakash, S., and Prakash, L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2635–2639
23. MacArthur, M. W., and Thornton, J. M. (1991) J. Mol. Biol. 218, 397–412
24. Pickart, C. M., and Rose, I. A. (1985) J. Biol. Chem. 260, 1573–1581
25. Hershko, A., Heller, H., Eytan, E., and Reiss, Y. (1986) J. Biol. Chem. 261, 11992–11999