Glucose and Ras Activity Influence the Ubiquitin Ligases APC/C and SCF in *Saccharomyces cerevisiae*

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ABSTRACT

In budding yeast, the Ras/cAMP pathway is involved in the coordination of cell growth and cell division. Glucose-rich medium stimulates Ras/cAMP signaling, which causes an increase in the critical cell size for cell cycle entry. Here we show that glucose and activated Ras proteins also influence the function of the anaphase-promoting complex (APC/C), a ubiquitin-protein ligase required for sister chromatid separation and mitotic exit. We found that *apc10-22* and other mutants defective in the APC/C are suppressed by reduced Ras signaling activity, by a deletion of the *RAS2* gene, by a *cdc25* mutation, by elevated levels of *PDE2*, or by growth without glucose. Viability of these mutants is also enhanced by decreased Cdk1 activity. In contrast, a constitutively activated $RAS2^{Val19}$ allele or shifts to glucose medium are deleterious to *apc10-22* mutants. Remarkably, *cdc34-2* mutants, which are impaired in SCF function, are differently affected with respect to Ras activity. Viability of *cdc34-2* mutants at elevated temperatures is dependent on glucose and the *RAS2* gene. We conclude that glucose and Ras proteins influence the APC/C and the SCF complex in an opposite manner. These ubiquitin ligases might represent novel targets for modulating cell division in response to growth conditions.

REGULATED proteolysis is a fundamental process in cell cycle progression of eukaryotic cells. Degradation of various target proteins is important for crucial events such as the initiation of DNA replication, the separation of sister chromatids during mitosis, or the inactivation of cyclin-dependent kinases (Cdks) prior to mitotic exit (reviewed by Hershko 1997). Protein degradation is normally preceded by the ligation of chains of ubiquitin molecules. The crucial components for the regulation of ubiquitination during the cell cycle are ubiquitin-ligase complexes, the Skp1/Cdc53/F-box complex (SCF), and the anaphase-promoting complex (APC/C), also known as cyclosome (reviewed by Peters 1998; Townsley and Ruderman 1998).

Different SCF complexes, which all require the ubiquitin-conjugating enzyme Cdc34 but vary in a subunit, the F-box protein (Bai *et al.* 1996; Skowyra *et al.* 1997), exist. SCF associated with the F-box protein Cdc4, SCF^{CDC4}, mediates proteolysis of the Cdk1 inhibitor Sic1, which is an essential process for the initiation of DNA replication (Schwob *et al.* 1994; Feldman *et al.* 1997; Verma *et al.* 1997). Several additional substrates of the SCF, such as Gcn4, Cdc6, and Far1, have been identified (Peters 1998). The SCF complex is active throughout the cell cycle, but the access of its substrates to the ubiquitination machinery is tightly controlled.

In contrast to SCF, the activity of the APC/C is cell cycle regulated (Cohen-Fix and Koshland 1997). Precise control of this highly conserved ubiquitin-protein ligase complex is a fundamental process ensuring a proper order of events during mitosis (Morgan 1999). One of the factors involved in APC/C activation at the metaphase to anaphase transition is the mitotic cyclin-dependent kinase, Cdk1. It is unknown whether Cdk1 phosphorylates the APC/C directly or whether it induces a pathway that leads to APC/C activation (Felix *et al.* 1990; Lahav-Baratz *et al.* 1995). Intriguingly, the yeast Cdk1, also known as Cdc28 kinase, is not only involved in promoting APC/C activation, but also acts as an inhibitor of the APC/C, at least during S-, G2-, and early M-phase (Amon 1997). Other crucial factors for APC/C activation during mitosis are Cdc20 and Cdh1/Hct1, which are two similar WD40 repeat proteins. Both proteins associate with the APC/C and are responsible for its substrate specificity (Schwab *et al.* 1997; Visintin *et al.* 1997; Fang *et al.* 1998). Cdc20 is needed for ubiquitination of the anaphase-inhibitor protein Pds1 and is therefore essential for the metaphase/anaphase transition (Visintin *et al.* 1997; Shirayama *et al.* 1998). Pds1 inhibits sister chromatid separation by blocking the "sister-separating" protein Esp1, thereby preventing the dissociation of sister chromatid cohesion molecules from chromosomes (Michaelis *et al.* 1997; Ciosk *et al.* 1998; Nasmyth 1999).

Proteolysis of mitotic cyclins in late anaphase requires the association of the APC/C with Cdh1/Hct1, which then enables the access of mitotic cyclins and other substrates like the spindle-associated protein Ase1 to the ubiquitination machinery (Schwab *et al.* 1997; Visintin *et al.* 1997). Binding of Cdh1/Hct1 to the APC/C

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was shown to be negatively regulated by Cdk1-dependent phosphorylation (Zachariae *et al.* 1998a; Jaspersen *et al.* 1999). A number of additional proteins, including Cdc5, Cdc14, Cdc15, Tem1, and Dbf2, are required for the exit from mitosis (reviewed by Morgan 1999). Most of these factors, designated as late mitotic proteins, are essential, display multiple genetic interactions, and, thus, probably have a function in a common pathway (Jaspersen *et al.* 1998). Cdc14 dephosphorylates Cdh1/Hct1 and thereby enables its association with the APC/C (Visintin *et al.* 1998; Jaspersen *et al.* 1999).

Other important factors in late mitosis were identified as the Ras genes *RAS1* and *RAS2*, as well as the Ras-related *RSR1* gene, which have a redundant but essential role in late mitosis (Morishita *et al.* 1995). Activation of the small GTP-binding proteins Ras1 and Ras2 normally stimulates adenylate cyclase, resulting in increased intracellular cAMP levels (Toda *et al.* 1985). However, the late mitotic function of the Ras proteins is apparently independent of the cAMP pathway (Morishita *et al.* 1995). In fission yeast, it was found that elevated levels of cAMP inhibit the APC/C (Yamashita *et al.* 1996). This inhibitory effect is mediated by protein kinase A (PKA) activity and might affect the assembly of the *Schizosaccharomyces pombe* APC/C (Yamashita *et al.* 1996; Yamada *et al.* 1997). PKA was also shown to phosphorylate the mammalian APC/C in an *in vitro* reconstituted system, thereby causing an inhibition of its activity (Kotani *et al.* 1998).

It might reflect an apparent discrepancy that Ras proteins are inducers of the cAMP pathway, which may interfere with APC/C activity, and that they simultaneously have an essential role for triggering mitotic exit. Here, we analyzed the effect of Ras activity on APC/C function in *Saccharomyces cerevisiae*. Initial experiments showed that *apc10-22* mutants, which are defective in APC/C-mediated proteolysis, are inviable at elevated temperatures on glucose medium, a rich medium known to induce the Ras/cAMP pathway. In contrast, these mutants were viable on a poor carbon source. We show that the deleterious effect of glucose on *apc10-22* and on other mutants in the APC/C occurs by the activity of Ras2 and cAMP. Thus, activated Ras signaling appears to inhibit the function of the APC/C. In contrast, we found that viability of mutants impaired in the SCF complex was enhanced by activated Ras signaling. Therefore, glucose and an activated Ras/cAMP signaling pathway influence the function of the APC/C and the SCF in opposite manners.

MATERIALS AND METHODS

Yeast strains and plasmids: The yeast strains used in this study are all derivatives of the W303 strain (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3 GAL psi*+). All mutants from different strain backgrounds were backcrossed at least four times to W303 strains to make them congenic. The *apc10-22* mutant was previously identified in a screen for mutants defective in cyclin proteolysis, but then incorrectly described as a *cse1-22* mutant (Irniger *et al.* 1995). The high-copy *SIC1* plasmid and the *GAL-PDS1-HA3* construct were obtained from M. Shirayama. *PDS1-HA3* contains three HA epitopes at position 520 after the first ATG of the *PDS1* open reading frame (ORF; Cohen-Fix *et al.* 1996). The high-copy *PDE2* plasmid and the mutated $RAS2^{Val19}$ allele were provided by H. U. Mösch. $RAS2^{Val19}$ was isolated as a 3.0-kb *Eco*RI-*Eco*RI fragment and was subcloned into the centromeric plasmid YCplac33 (Gietz and Sugino 1988). For disruption of the *RAS2* gene, a disruption cassette with a complete deletion of the *RAS2* ORF was used. The 1.16-kb *Hpa*I-*Hpa*I fragment was replaced by the *URA3* gene. After transformation of yeast cells, disruptions were verified by Southern hybridization.

Genetic techniques and media: Standard genetic techniques were used for manipulating yeast strains. To test synthetic phenotypes, the corresponding haploid strains were crossed, the diploids were sporulated, and the resulting tetrads were analyzed by dissection. Only tetrads producing four germinating spores were used for the analysis of genetic interaction.

Mutant strains that contained two temperature-sensitive alleles, *apc10-22 cdc25-1*, *cdc27-1 cdc4-1*, *cdc27-1 cdc34-1*, and *cdc23-1 cdc4-1*, were obtained by crossings of haploid strains and tetrad analysis. Segregants from nonparental ditype tetrads were used. In each case, these strains were retested by backcrossing to a wild-type strain, thereby verifying the presence of two temperature-sensitive alleles.

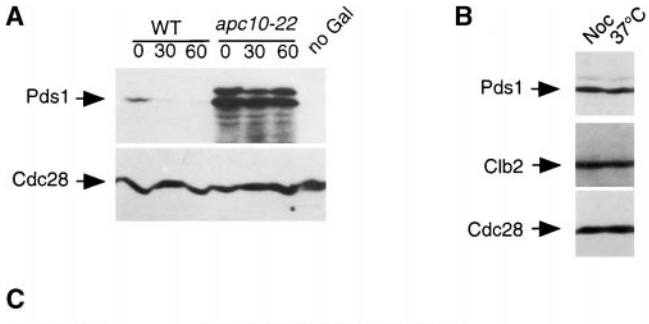
When yeast cells were grown in complete medium, YEP medium (2% bactopeptone, 1% yeast extract, 0.005% adenine sulfate) supplemented either with 2% glucose (YEPD) or 2% raffinose (YEP + Raff) was used. For the selection of plasmid-containing strains, cells were grown in minimal medium, a synthetic medium containing 0.8% yeast nitrogen base without amino acids, 50 μg/ml uracil and adenine, supplemented with amino acids and 2% glucose or raffinose (Rose *et al.* 1990).

Growth conditions and cell cycle arrests: Prior to the incubation of mutant strains at elevated temperature on agar plates, the cells were always preincubated at 25° for 12–18 hr. Prior to cell cycle arrests in liquid medium, cultures were pregrown to an OD_{600} 0.3–0.6 at 25°. When a gene was expressed from the inducible *GAL1* promoter, cells were pregrown in medium containing raffinose as the sole carbon source. The *GAL1* promoter was induced by the addition of 2% galactose. To arrest cells in G1-phase with α-factor pheromone, 0.5 μg/ml α-factor was added to *bar1* strains. For prolonged α-factor treatments, additional α factor (0.25 μg/ml) was added after every 120 min to prevent a drop in the α-factor concentration. To arrest cells with the microtubule-depolymerizing drug nocodazole, 15 μg/ml nocodazole (from a 1.5 mg/ml stock solution in DMSO) was added.

Other methods: Whole cell extracts for immunoblotting were prepared as previously described (Surana *et al.* 1993). Immunoblotting was performed using the enhanced chemiluminescence detection system (ECL; Amersham). Clb2 and Cdc28 antibodies were used in 1:1000 and 1:2000 dilutions, respectively. To detect HA-tagged proteins, the HA antibody (12CA5) was used in a 1:100 dilution. For immunofluorescence microscopy, cells were fixed in 3.7% formaldehyde and subsequently spheroplates were prepared as described by Pringle *et al.* (1991). 4′,6-Diamidino-2-phenylindole (DAPI) staining and antitubulin antibodies were used for visualization of nuclei and spindles, respectively.

RESULTS

Apc10 is generally required for ubiquitin-dependent proteolysis mediated by the anaphase-promoting complex: Apc10 (also termed Doc1) was previously shown to be essential for proteolytic degradation of the mitotic



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Genotype	Number of viable segregants
APC10 UBC4	57
APC10 ubc4::HIS3	66
apc10-22 UBC4	60
apc10-22 ubc4::HIS3	0

Figure 1.—*apc10-22* mutants are defective in APC/C-mediated proteolysis. (A) Wild-type cells and *apc10-22* mutants, both containing a *bar1::hisG* deletion and a *GAL1-PDS1* fusion (*PDS1* tagged with three HA epitopes), were arrested at 25° with α-factor pheromone. Subsequently, galactose was added to induce the *GAL1* promoter for 30 min, then the cells were shifted to 36° for 30 min, and finally glucose was added to turn off transcription of the fusion gene. Samples were collected 0, 30, and 60 min after glucose addition. Pds1 stability was determined by immunoblotting with an antibody to the HA epitope. Pds1 often appeared as a doublet, as described by Cohen-Fix *et al.* (1996). Cdc28 was used as a loading control. (B) *apc10-22* mutants containing an HA-tagged version of Pds1 were incubated either with nocodazole at the permissive temperature (25°) or without nocodazole at the restrictive temperature (37°). Samples were collected after 4 hr and were analyzed by immunoblotting. (C) Tetrad analysis of a cross of *apc10-22* mutants with an *ubc4::HIS3* deletion strain. A total of 60 tetrads, of which all four spores were germinated, were analyzed.

cyclin Clb2 (Hwang and Murray 1997). Apc10 was found to be associated with the anaphase-promoting complex in the budding yeast *S. cerevisiae* (Hwang and Murray 1997; Zachariae *et al.* 1998b) and the fission yeast *S. pombe* (Kominami *et al.* 1998). Findings in *S. pombe* indicated that Apc10 is a regulator of the APC/C and possibly is not a subunit of this complex.

APC/C activity is primarily regulated by association with its substrate-specific activators Cdc20 and Cdh1/Hct1. To elucidate whether Apc10 is generally needed for APC/C activity or whether it is involved in substrate-specific APC/C activation, the stability of a noncyclin substrate, the anaphase inhibitor Pds1, was determined in a temperature-sensitive *apc10-22* budding yeast mutant. Pds1 was stabilized in these cells during G1-phase, under conditions where the APC/C is fully active in wild-type cells (Figure 1A). Furthermore, *apc10-22* mutant arrested at the restrictive temperature in mitosis with high Pds1 levels, comparable to cells blocked in metaphase by nocodazole treatment (Figure 1B). Therefore, Apc10 appears to be generally required for APC/C function. A direct role for Apc10 in ubiquitination is further supported by genetic crossing of an *apc10-22* mutant and a strain lacking the ubiquitin-conjugating enzyme Ubc4. Segregants containing both mutations were inviable at 25°, implying that a combination of these mutations results in a synthetic lethal phenotype (Figure 1C).

***apc10-22* mutants are suppressed by decreasing Ras signaling and cAMP levels:** To identify putative regulators of the APC/C, we used the temperature-sensitive *apc10-22* mutant to screen for suppressors of this mutation. During screenings of cDNA libraries, we observed that the *apc10-22* mutants had a distinctly different phenotype when grown on media containing raffinose or galactose compared to glucose medium. This effect was independent of plasmids from the cDNA libraries. Most cells incubated on medium containing glucose (YEPD) were inviable at 35° (Figure 2A). Only a few cells were capable of undergoing cell division and eventually producing colonies. In contrast, most *apc10-22* mutant cells produced colonies on agar plates containing the poor carbon source raffinose. It was shown earlier that the addition of glucose to poor growth medium activates the Ras/cAMP signaling pathway, leading to a transient, drastic increase in cAMP levels, followed by a resetting to a level that is modestly higher than before glucose addition (Russell *et al.* 1993; Tokiwa *et al.* 1994). The inhibitory effect of glucose on *apc10-22* mutants might therefore be mediated by the activation of the Ras signaling pathway and increased cAMP levels.

To test this hypothesis, we constructed various *apc10-22* mutant strains in which Ras signaling was downregulated, either by deleting the *RAS2* gene or by the combination of *apc10-22* with a *cdc25-1* mutation, a mutant in the guanine nucleotide exchange factor for Ras. Both the *apc10-22 ras2*Δ and the *apc10-22 cdc25-1* double mutants were viable at 35° on YEPD plates (Figure 2, B and C). Colonies grew more slowly than wild-type colonies, but cells had a morphological phenotype similar to wild-type cells (data not shown). In contrast, *apc10-22* cells increased rapidly in cell size and ceased cell division as large budded cells. To test if the inhibitory effect of glucose occurs via the cAMP pathway, *apc10-22* was transformed with a high-copy plasmid containing the cAMP-phosphodiesterase gene *PDE2*, which is known to reduce cAMP levels. Overexpression of *PDE2* had an effect similar to that of Ras2 inactivation, indicating that low cAMP levels restored viability to *apc10-22* mutants (Figure 2D).

Taken together, these results show that decreasing Ras activity or cAMP levels suppressed the lethality of *apc10-22* mutants. Thus, glucose and Ras activity are apparently inhibitors of Apc10 function.

Activation of Ras signaling blocks cell division of *apc10-22* mutants: To test whether activated Ras signaling is deleterious to *apc10-22* mutants, a plasmid containing a constitutively activated *RAS2* allele, $RAS2^{Val19}$, was transformed into *apc10-22* mutants. Only a low number of transformants were able to grow at 25° and these exhibited growth defects even at this permissive temperature. Upon incubation of these cells at 35° on medium containing the poor carbon source raffinose, most cells containing the activated *RAS2* allele were inviable (Fig-

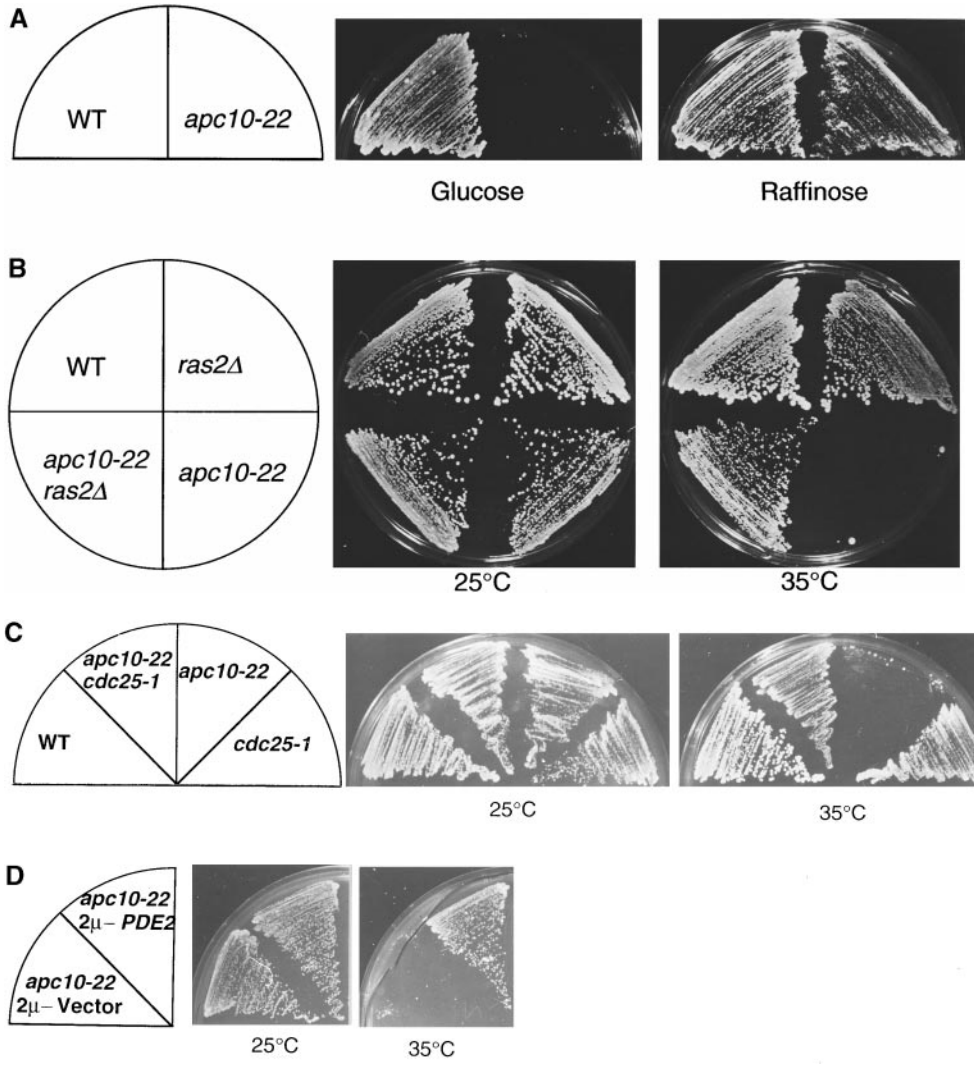

Figure 2.—Suppression of the lethality of *apc10-22* mutants by decreasing Ras signaling. (A) Wild-type cells and *apc10-22* mutants were streaked on YEP agar plates containing either glucose or raffinose as the sole carbon source. Plates were incubated at 35° for 2.5 days. On galactose plates, most cells formed colonies like on raffinose plates (not shown). (B) Wild-type cells, a *ras2::URA3* deletion strain (*ras2*Δ), an *apc10-22* mutant, and an *apc10-22 ras2*Δ double mutant were incubated on YEPD medium for 2.5 days. (C) Wild-type cells, an *apc10-22 cdc25-1* double mutant, and the corresponding single mutants were incubated on YEPD plates for 2.5 days. (D) *apc10-22* mutants containing high-copy plasmids, either with the *PDE2* gene (2μ-*PDE2*) or with no insert (2μ-Vector), were incubated on minimal medium plates containing 2% glucose at the indicated temperatures for 3 days.

ure 3A). These cells increased rapidly in cell size and ceased cell division, whereas strains containing a control plasmid were able to form colonies (Figure 3B). Therefore, *apc10-22 RAS2*^{Val19} cells growing on raffinose medium had a similar phenotype to *apc10-22* cells growing on glucose medium, whereas the *apc10-22* control strain was morphologically similar to the *apc10-22 ras2*Δ and *apc10-22 cdc25-1* strains shown in Figure 2. This observation demonstrates that an activated Ras2 protein is deleterious to *apc10-22* mutants.

To demonstrate the inhibitory effect of glucose on cell division of *apc10-22* mutants, glucose was added to cultures that were preincubated in raffinose medium. Under these conditions, *apc10-22* mutants were able to divide similarly to wild-type cells at temperatures up to 32°. The addition of 2% glucose resulted in the accumulation of large budded cells that contained mostly short mitotic spindles (Figure 4A). This phenotype was not observed when galactose was added instead of glucose. To further confirm that these cells were indeed blocked in mitosis, α-factor pheromone was added, which normally causes dividing cells to arrest in G1-phase. During a 5-hr period following α-factor addition, only 20–25% of cells incubated in glucose medium arrested as unbudded cells, whereas in the culture containing galactose and in the wild-type control cultures, >90% of the cells entered G1-phase (Figure 4B). Glucose prevented a decrease in the levels of the APC/C substrates Pds1 and Clb2 during this experiment (Figure 4C). Protein levels decreased, but did not disappear completely when *apc10-22* mutants were incubated with galactose. In this case, APC/C may be only partially functional, but sufficiently functional to exit mitosis despite the presence of low levels of Pds1 and Clb2.

These experiments show that glucose induces a mitotic arrest of *apc10-22* mutants by blocking APC/C function, thereby preventing Pds1 and Clb2 degradation.

Decreasing Ras signaling suppresses *cdc27-1*, *cdc23-1*, and *dbf2-2* mutants: To test whether the suppression of *apc10-22* by low Ras activity is a specific phenomenon for this mutant, we compared various temperature-sensitive cell cycle mutants on medium containing glucose *vs.* medium containing raffinose. Many of these mutants, including mutants in the yeast Cdk1, *cdc28-1N* and

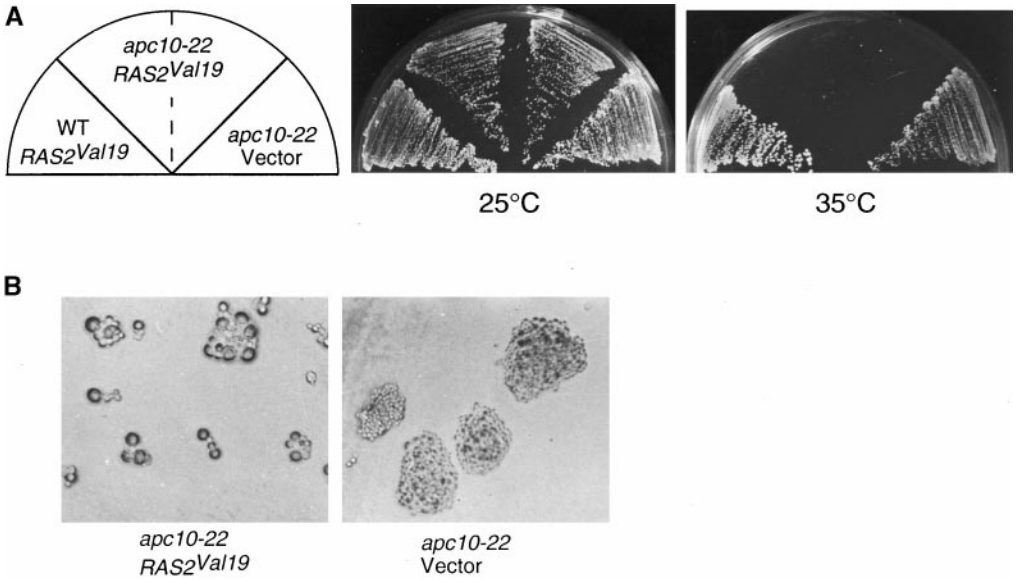

Figure 3.—Activation of Ras signaling is toxic for *apc10-22* mutants. (A) *apc10-22* transformed with centromeric plasmids containing either a $RAS2^{Val19}$ gene or no insert were streaked on YEP medium containing raffinose as the sole carbon source. (B) Photographs of cells incubated on YEP + Raff at 35°, 20 hr after streaking and temperature shift.

cdc28-4, showed no obvious difference when grown either on a poor or on a rich carbon source (not shown). Nevertheless, additional mutants whose viability was enhanced by incubation on poor carbon sources were identified. *cdc27-1* mutants, which are defective in an APC/C subunit, were inviable at 30° on glucose medium, but divided and formed colonies when incubated on raffinose medium (Figure 5A) or in the presence of high levels of *PDE2* (not shown).

Suppression of another mutant in the APC/C, *cdc23-1*, was visible, but was less distinct than for *cdc27-1* mutants (not shown). However, we found that growth in the absence of glucose restores viability to *cdc23-1 clb2* double mutants, mutations that were previously found to be synthetic lethal (Figure 5B; Irniger *et al.* 1995). Whereas spores containing both the *cdc23-1* mutation and a deletion of the mitotic cyclin gene *CLB2* were dead on YEPD medium, many of them were able to produce colonies on glucose-free medium at 25°. Plating to YEPD plates confirmed that the lethality of *cdc23-1 clb2* cells is due to the presence of glucose (Figure 5C).

It is likely that *cdc23-1* cells lacking *CLB2* are defective in APC/C activation induced by the mitotic Cdk1. This strong effect of glucose on the *cdc23-1 clb2* double mutant, as well as the finding that glucose caused *apc10-22* mutants to arrest with short mitotic spindles (Figure 4A), may indicate that Ras signaling specifically inhibits APC/C activation at the metaphase/anaphase transition. To test whether mitotic exit is similarly affected by Ras activity, we used *cdc23-1* cells in which *CLB2* was modestly expressed to higher levels, caused by an *ADH-CLB2* fusion construct. *cdc23-1* cells expressing *CLB2* at elevated levels arrest with elongated spindles and fail to exit from mitosis (Irniger *et al.* 1995). We found that modestly elevated *CLB2* expression was more deleterious to *cdc23-1* cells grown on glucose medium than to cells incubated on raffinose medium (Figure 5D). Thus, Ras activity also affects *cdc23-1* cells, which are primarily defective in mitotic exit. From these observations, we conclude that Ras signaling generally affects the function of the APC/C and not specifically its activation in early anaphase.

Furthermore, *dbf2-2* mutants that are defective in mitotic exit exhibit phenotypes similar to those of mutants in the APC/C. The lethality of *dbf2-2* cells is suppressed efficiently by a *ras2* deletion and modestly by high levels of *PDE2* (Figure 5E). Like *apc10-22* mutants, *dbf2-2* was sensitive to an activated $RAS2^{Val19}$ allele (not shown). Therefore, Ras activity affects not only APC/C function, but also additional factors important for mitotic exit.

Synergistic effect of decreasing Ras and Cdk1 activity: It is known that Cdk1 is required to keep the APC/C inactive during S- and G2-phase and in nocodazole-arrested mitotic cells (Amon 1997). Therefore, Cdk1 seems to act as an inhibitor of the APC/C. To compare the effect of Cdk1 activity on APC/C function with the effect of Ras activity, we tested whether decreasing Cdk1 activity also affects the viability of *apc10-22* and *cdc27-1* mutants. Clb-associated Cdk1 activity is inhibited efficiently by the CDK inhibitor Sic1 (Schwob *et al.* 1994) and expression of *SIC1* on a high-copy plasmid is likely to result in a reduction of Cdk1 activity. Indeed, we found that elevated levels of Sic1 suppressed the lethality of *apc10-22* and *cdc27-1* mutants at elevated temperature (Figure 6, A and B). Remarkably, *apc10-22* mutants were suppressed efficiently at up to 37° on YEPD medium. These results show that decreasing Cdk1 activity enhances viability of mutants impaired in APC/C function and suggest an inhibitory effect of Cdk1 on the APC/C.

It might be assumed that suppression of *apc10-22* and *cdc27-1* mutants by reduced Cdk1 activity is due to an alleviated association of Cdh1/Hct1 with the APC/C. Binding of Cdh1/Hct1 is negatively regulated by Cdk1 activity (Zachariae *et al.* 1998a; Jaspersen *et al.* 1999).

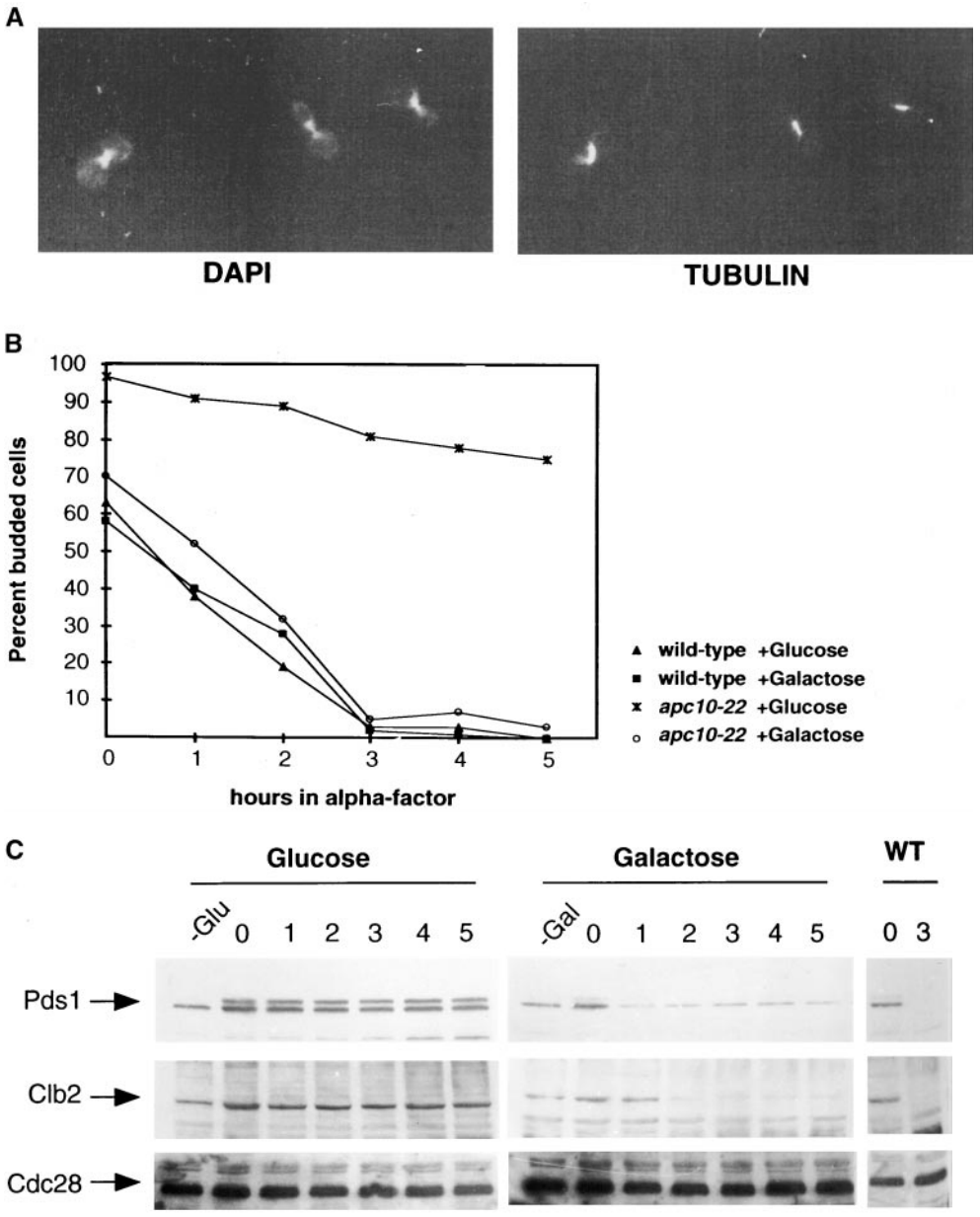

Figure 4.—Glucose blocks *apc10-22* cells in mitosis. Wild-type and *apc10-22* mutant cells were pregrown at 32° in YEP + Raff medium to a low exponential growth phase (OD 0.1 to OD 0.2). Under these conditions, mutant cells divided without an obvious growth defect, similar to wild-type cells. The culture was split and subsequently either 2% glucose or 2% galactose was added. Incubation continued for another 5 hr at 32°. Then α-factor pheromone was added to elucidate whether cells are capable of undergoing mitosis and then arrest in G1-phase as a consequence of the pheromone treatment. (A) Photographs of *apc10-22* mutants 5 hr after glucose addition. DNAs and spindles were visualized by DAPI staining or tubulin antibodies, respectively. A total of 80–90% of the cells contained short spindles, whereas most of the remaining cells had elongated spindles. (B) Percentage of budded cells, determined every hour after the addition of α-factor (0 time point). (C) Immunoblotting of cell extracts collected every hour after the addition of α-factor (0 time point). Strains containing Pds1 tagged with three HA epitopes were used for these experiments (Cohen-Fix *et al.* 1996). Pds1 and Clb2 were detected with HA or Clb2 antibodies, respectively. Pds1 often appeared as a doublet. Cdc28 was used as a loading control. Samples without glucose (−Glu) or galactose (−Gal) were collected prior to glucose and galactose addition, respectively. For wild-type strains, a sample prior to α-factor addition (0 min) and a sample after 3 hr of α-factor treatment are shown.

However, suppression of *apc10-22* mutants by low kinase cannot be due solely to elevated binding capacity of Cdh1/Hct1, because an *apc10-22* mutant strain in which *CDH1/HCT1* was deleted was also viable at 37° when containing high levels of the Sic1 protein (not shown).

We have shown that a reduction in either Cdk1 kinase activity or Ras/cAMP signaling restored viability of *cdc27-1* cells at 30° (Figures 5A and 6B), but never at temperatures above 32°. This partial phenotype allowed us to test whether combined downregulation of Ras and Cdk1 kinase results in a synergistic effect. To elucidate this, a *cdc27-1* strain containing a high-copy *SIC1* plasmid was incubated at elevated temperatures. At 35°, this strain was viable on raffinose plates, whereas on glucose medium most cells were inviable (Figure 6C). Therefore, viability of *cdc27-1* mutants at 35° is dependent both on reduced Cdk1 kinase activity and on low Ras activity. This synergistic effect on *cdc27-1* mutants suggests that Cdk1 kinase and the Ras signaling pathway act in parallel to inhibit APC/C function.

Viability of *cdc34-2* and *cdc4-1* mutants depends on active Ras signaling: Remarkably, we found that two

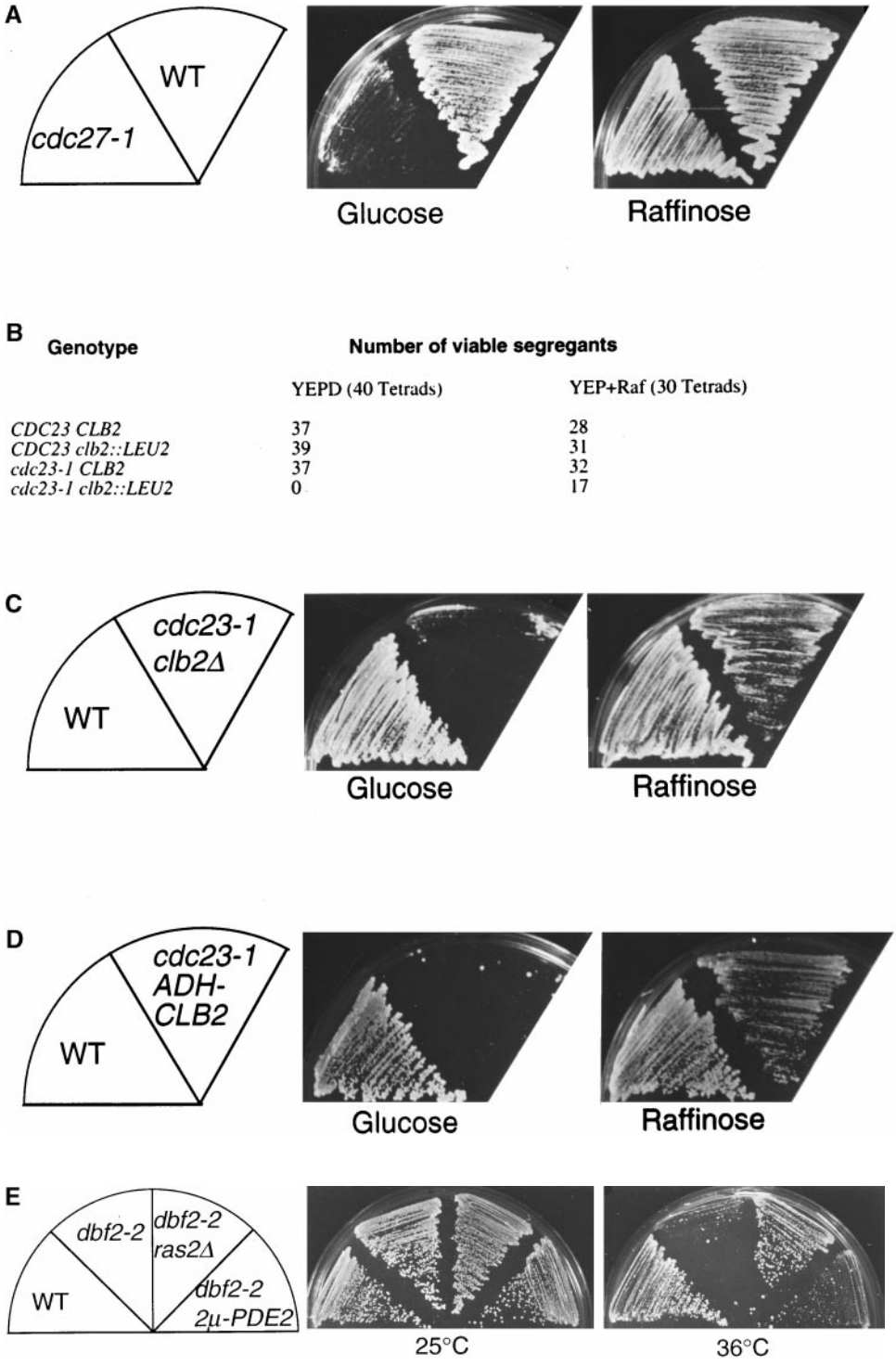

Figure 5.—Viability of *cdc27-1*, *cdc23-1*, and *dbf2-2* mutants is enhanced by a reduction in Ras activity. (A) Wild-type and *cdc27-1* cells were incubated at 30° for 2.5 days. (B) Tetrad analysis of a cross of *cdc23-1* cells with a *clb2* deletion strain (*clb2::LEU2*). Tetrads were dissected on complete medium containing either glucose or raffinose and were incubated at 25°. (C) *cdc23-1 clb2::LEU2* segregants were streaked on YEPD or YEP + Raff medium and were incubated at 25° for 3.5 days. (D) *cdc23-1* cells in which Clb2 is modestly overexpressed by the *S. pombe ADH1* promoter (*ADH-CLB2*) were incubated either on glucose or raffinose medium at 28° for 3 days. (E) Wild-type cells, *dbf2-2* mutants, and *dbf2-2* cells with a *ras2* deletion were incubated on YEPD agar plates at the indicated temperatures for 2.5 days. In addition, *dbf2-2* transformants containing the *PDE2* gene on a high-copy vector were streaked on these plates. Viability of *cdc15-2* mutants was also modestly enhanced by a decrease in Ras activity (not shown).

mutant strains, *cdc34-2* and *cdc4-1*, displayed opposite phenotypes with respect to glucose and Ras activity when compared to *apc* mutants. *cdc34-2* and *cdc4-1* mutants are both defective in the SCF complex. Viability of these mutant strains was enhanced when cells were grown in the presence of glucose. At elevated, semipermissive temperatures, *cdc34-2* mutants were able to produce normal colonies on YEPD plates, but were severely impaired on medium containing raffinose (Figure 7A). Inactivation of *RAS2* was deleterious to *cdc34-2* mutants on glucose medium and these cells were completely inviable on raffinose medium.

The synthetic phenotype of a *ras2* deletion and *cdc34-2* mutation might be explained by the fact that both Ras and SCF are needed for the G1/S transition. Ras proteins are essential in late G1-phase for the stimulation

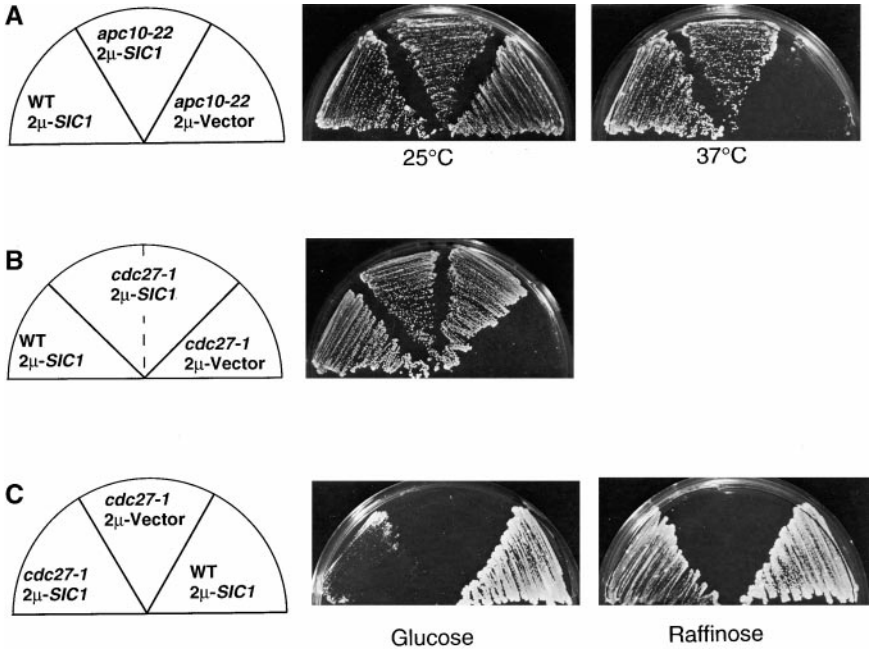

Figure 6.—High levels of Sic1 enhance viability of mutants in the APC/C. (A) *apc10-22* mutants containing either the *SIC1* gene on a high-copy plasmid or the empty vector plasmid (YEplac181) were incubated for 2.5 days at the indicated temperatures on YEPD agar plates. Overexpression of *SIC1* had no effect on wild-type cells. (B) *cdc27-1* mutants containing either the *SIC1* gene on a high-copy plasmid (two transformants are shown) or the empty vector plasmid were incubated for 2.5 days at 30°. (C) *cdc27-1* mutants containing either the *SIC1* gene on a high-copy plasmid or the empty vector plasmid were incubated for 2.5 days at 35° on medium containing either glucose or raffinose as the sole carbon source.

of adenylate cyclase (Toda *et al.* 1985), whereas the SCF complex triggers the degradation of the Cdk1 inhibitor Sic1 at the G1/S transition (Schwob *et al.* 1994). We therefore tested whether the failure to degrade Sic1 is the sole reason for the lethality of *cdc34-2* mutants under conditions where Ras signaling activity is low. We constructed a *cdc34-2 sic1*Δ mutant strain that is able to undergo a complete round of DNA replication (Schwob *et al.* 1994). This strain was incubated on glucose and raffinose medium. We found that this strain is viable on glucose medium but inviable on raffinose medium at elevated temperatures (Figure 7B). Therefore, viability of both *cdc34-2* and *cdc34-2 sic1*Δ mutants depends on active Ras signaling.

In *cdc4-1* mutants, the influence of the carbon source was less significant than in *cdc34-2* mutants (Figure 7C). Nevertheless, under conditions where Ras signaling is low, by incubation of a *cdc4-1 ras2*Δ strain on raffinose medium, cells were inviable at 30°. Because the viability of *cdc34-2*, and to a lesser extent of *cdc4-1* cells, at ele-

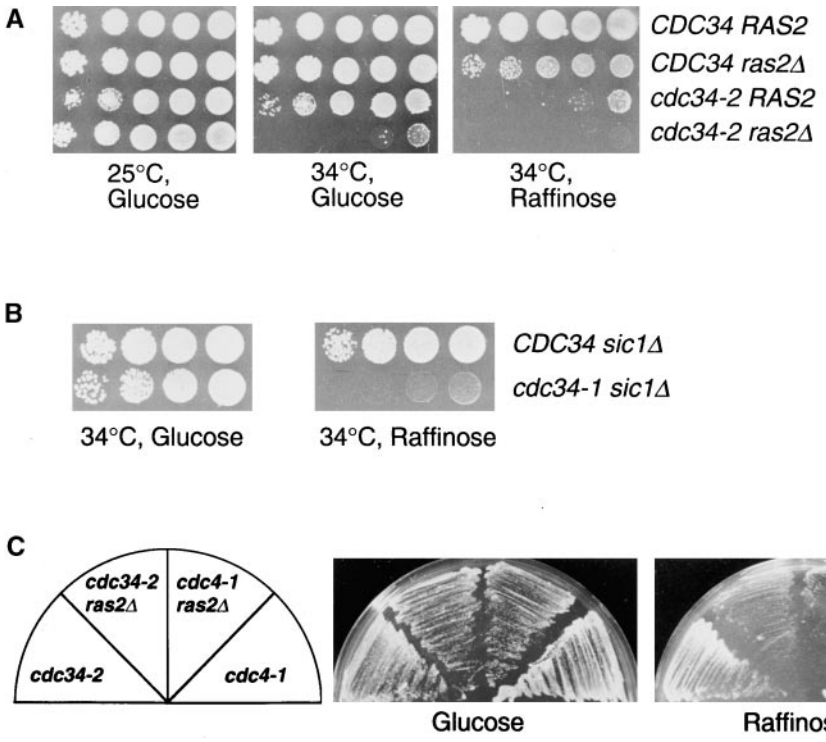

Figure 7.—Viability of *cdc34-2* and *cdc4-1* mutants is dependent on glucose and Ras2. (A and B) Serial dilutions (1:10) of the indicated strains were spotted on agar plates (from right to left) containing the indicated carbon source and were incubated for 3 days at 25° or 34°. (C) *cdc34-2* and *cdc4-1* mutants, as well as the mutant strains containing a deletion of the *RAS2* gene, were incubated on agar plates containing the indicated carbon source and were incubated at 30° for 3 days.

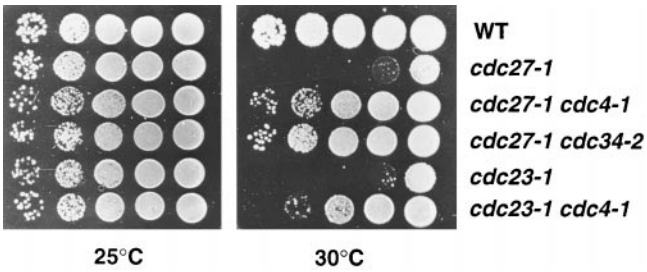

Figure 8.—Partial inactivation of SCF suppresses the lethality of mutants in the APC/C. Serial dilutions (1:10) of the indicated mutant strains were spotted on YEPD plates (from right to left). Plates were incubated for 3 days at the indicated temperatures.

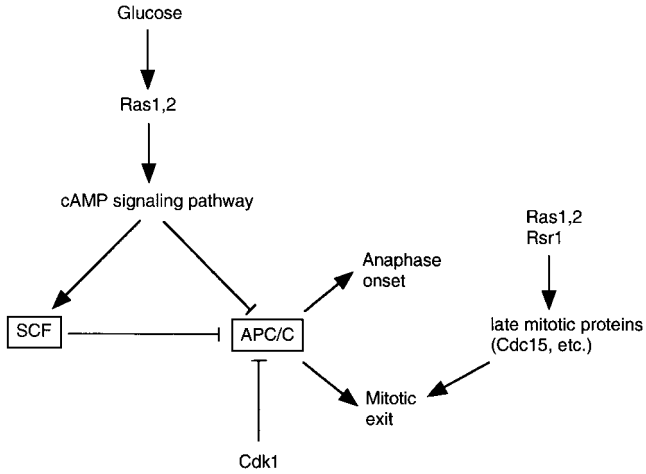

Figure 9.—Model for Ras-mediated regulation of SCF, APC/C, and mitotic exit. Glucose stimulates Ras1 and Ras2 proteins, which activate adenylate cyclase and cause an increase in cAMP levels. Activation of this pathway enhances SCF function, but inhibits the APC/C. It is not known yet whether these effects are directly mediated by PKA. Glucose, a rich medium, may transiently delay mitotic events like sister chromatid separation (anaphase onset) and mitotic exit and therefore may allow cells to grow to a larger cell size before cell division. Ras1, Ras2, and the Ras-related protein Rsr1 have an essential role in triggering mitotic exit, which seems to be independent of cAMP and PKA, but appears to be connected to late mitotic proteins such as Cdc15 (Morishita *et al.* 1995). Like the Ras/cAMP pathway, SCF and Cdk1 activity negatively influence APC/C function.

vated temperatures is dependent on Ras activity, we conclude that SCF function is enhanced by active Ras signaling.

Partial inactivation of SCF suppresses defects in the APC/C: Our data imply that the two ubiquitin-ligase complexes SCF and APC/C are both influenced by the activity of the Ras pathway, either positively or negatively. An increase or decrease in Ras signaling might be part of a mechanism to promote one proteolytic pathway and to reduce the second one. Our findings prompted us to identify possible genetic interaction between these complexes. For this purpose, mutant strains containing temperature-sensitive mutations in both the SCF and the APC/C complexes were constructed. We used *cdc23-1* and *cdc27-1* mutants that are inviable at 30° on YEPD and combined them with *cdc4-1* or *cdc34-2* mutants that are viable under these conditions. These double mutant strains allowed us to test the effect of mutations in SCF on mutants in the APC/C. We found that the lethality of *cdc27-1* mutant strains was suppressed when combined with mutations in the SCF (Figure 8). Suppression of the *cdc23-1* mutant also occurred, but it appeared to be less efficient. Therefore, partial inactivation of SCF suppresses defects in the anaphase-promoting complex. A reverse experiment under conditions where viability of *cdc34-2* mutants might be enhanced by mutations in the APC/C did not reveal any suppression of these mutants (not shown). These results indicate that SCF activity has an inhibitory effect on the APC/C.

DISCUSSION

Ras activity and mitosis: Precise regulation of the anaphase-promoting complex is crucial to guaranteeing a proper order of events during mitosis. Control mechanisms that regulate APC/C activity are therefore important prerequisites for faithful cell cycle progression in eukaryotic organisms. We have shown here that nutritional conditions and the activity of Ras signaling affect APC/C function in the budding yeast *S. cerevisiae* (Figure 9). Our results indicate that rich medium and activated Ras signaling have an inhibitory influence on APC/C function.

It is unknown whether this inhibition affects APC/C activity directly or indirectly. In fission yeast, it was found that the addition of cAMP was deleterious for *cut4* mutants defective in APC/C function. *cut4* and *cut9* mutants were suppressed by a high dosage of a cAMP phosphodiesterase gene or by a deletion of PKA (Yamashita *et al.* 1996; Yamada *et al.* 1997). It was concluded that elevated cAMP levels inhibited the assembly of the APC/C. In mouse fibroblasts, PKA directly phosphorylated subunits of the APC/C and thereby inhibited its activation (Kotani *et al.* 1998). Our studies on the influence of glucose, Ras activity, and cAMP on APC/C function in budding yeast extend these observations from other organisms, and we conclude that cAMP- and PKA-mediated inhibition of APC/C activity is a conserved mechanism in eukaryotes.

In budding yeast, Ras proteins were identified previously as essential components for the completion of M-phase (Morishita *et al.* 1995). Simultaneous disruption of *RAS1*, *RAS2*, and a *RAS*-related gene, *RSR1*, resulted in a late mitotic arrest. The late mitotic role of Ras proteins is obviously independent of the cAMP pathway, but is connected to the function of the late mitotic genes. Multiple copies of genes such as *CDC5*,

CDC15, and *DBF2* suppressed the lethality of the triple *RAS* deletion. This essential role of Ras proteins in triggering mitotic exit is apparently inconsistent with the findings that activation of Ras proteins inhibits APC/C function. Activated Ras proteins in late anaphase/telophase could interfere with APC/C function. But at this stage of mitosis, APC/C activity is important for B-type cyclin degradation, which results in Cdk1 inactivation, spindle depolymerization, and cytokinesis. In late mitosis, APC/C is also required for ubiquitination of additional proteins such as the spindle-associated protein Ase1 and the polo-like protein kinase Cdc5 (Pellman *et al.* 1995; Charles *et al.* 1998; Shirayama *et al.* 1998).

It is tempting to speculate that Ras proteins have a dual role during mitosis that may help to coordinate critical mitotic events. In anaphase, Ras activity and cAMP levels might be low, ensuring that the APC/C is fully active. Indeed, it was found that in mouse fibroblast cells PKA activity decreases rapidly in anaphase (Kotani *et al.* 1998) and that in budding yeast cAMP levels drop before cells undergo cell division (Smith *et al.* 1990). Inactive Ras during anaphase may ensure high APC/C activity, but may prevent late mitotic proteins like Cdc14 or Cdc15 from being activated before the APC/C has triggered essential processes such as sister chromatid separation and before chromosome segregation is completed. It is still unknown by which signals or mechanism late mitotic proteins are activated in late anaphase. A switch from inactive to active Ras proteins in late anaphase could be involved in the activation of the network of late mitotic proteins.

An indication for a regulator role of Ras proteins during mitosis is the identification of a *Xenopus laevis N-Ras* gene as a high-dosage suppressor of a yeast *cdc15-2* mutant (Spevak *et al.* 1993). Only the frog *N-Ras* gene, but not the yeast *RAS* genes, was capable of suppressing this mutant when overexpressed. Intriguingly, N-Ras was unable to activate adenylate cyclase, but rather interfered with its normal function. This resulted in unusually low cAMP levels, which allowed *cdc15-2* mutants to exit mitosis. In an independent screen, Yak1, a protein kinase that opposes PKA function (Garrett and Broach 1989; Garrett *et al.* 1991), was identified as a suppressor of several mutants in late mitotic genes, including *cdc15-2* (Jaspersen *et al.* 1998).

A possible link between the activities of Ras proteins and the APC/C might be Cdc25, a guanine nucleotide exchange factor for Ras (Broek *et al.* 1987). Cdc25 is an unstable protein containing a cyclin destruction box (Kaplon and Jacquet 1995). It is not clear whether this destruction box is indeed functional, but a truncated protein lacking a large N-terminal region was distinctly more stable than the wild-type protein. It also remains to be analyzed whether Cdc25 is a target of the APC/C and whether Cdc25 stability is regulated. APC/C-mediated proteolysis of Cdc25 might open the possibility of a feedback mechanism regulating APC/C and Ras activities. A reduction in Ras signaling might result in elevated APC/C activity and this in turn would enhance Cdc25 degradation and subsequently cause lower Ras activity.

Coordination of cell division and cell growth: Cell division needs to be coordinated with cell growth, which depends on environmental conditions, such as the availability of nutrients. The presence of rich carbon sources allows cells to increase in cell size and to propagate as larger cells compared to cells grown on poor medium. Previously, it was demonstrated that modulation of cell cycle progression in response to growth conditions occurs in late G1-phase (Johnston *et al.* 1979). An increase in intracellular cAMP levels induced by rich medium causes a transient G1 delay and, thus, the critical cell size to initiate budding is set to a larger value. It is unclear how during G1-phase the cAMP signal is transmitted to the cell cycle machinery, but the G1 cyclins *CLN1* and *CLN2* were identified as target genes (Baroni *et al.* 1994; Tokiwa *et al.* 1994). cAMP transiently reduces G1 cyclin transcription and thereby delays entry into S-phase.

It is unlikely that a coordination of cell cycle progression and cell growth occurs exclusively during G1-phase. A flow cytometric approach revealed that, upon a shift from ethanol to glucose medium, a fraction of cells was delayed in mitosis, resulting in newborn daughter cells of an increased cell size (Alberghina *et al.* 1998). This observation indicates a regulatory mechanism that allows the daughter cell to reach a certain critical size before cell division and, as a consequence, daughter cells are born at a larger cell size. Our results support the existence of a regulatory mechanism prior to cell division resulting in a transient mitotic delay. The inhibitory influence of glucose and Ras activity on APC/C function indicates that this ubiquitin-ligase complex is a putative target for modulating cell cycle progression in response to cell growth during mitosis.

The pseudohyphal growth of yeast cells as a consequence of nitrogen starvation is an example where the coordination of cell cycle progression and cell growth during G2/M-phase has an important physiological role. Cells exit mitosis and start rebudding only after the daughter cell has reached a cell size similar to the mother cell (Kron *et al.* 1994). This delay in G2/M-phase in pseudohyphal cells might be caused by a transient inhibition of the APC/C due to the activation of Ras signaling. Indeed, the Ras/cAMP signaling pathway is crucial for pseudohyphae formation (Gimeno *et al.* 1992; Mösch *et al.* 1996, 1999; Kübler *et al.* 1997). Remarkably, a screen for mutants defective in filamentous growth identified a *cdc55* mutant allele (Mösch and Fink 1997). A *cdc55* mutant was also found to suppress *cdc20-1* mutants and Cdc55, a phosphatase regulatory subunit, was identified as a component of the spindle assembly checkpoint (Wang and Burke 1997). Thus, Cdc55 is likely involved in the regulation of the

APC/C-Cdc20 complex and may link the pseudohyphal signaling pathway to the cell cycle machinery.

Coordination of the APC/C and SCF ubiquitin ligases: In contrast to the mutants in the APC/C, we found that viability of mutants impaired in SCF function was enhanced by activated Ras signaling. Growth in the absence of glucose or a deletion of the *RAS2* gene was deleterious to *cdc34-2* cells, implying that activated Ras signaling is required for proper SCF function.

It is unknown how Ras signaling promotes SCF activity. Ras1 and Ras2 are essential in late G1-phase for the stimulation of adenylate cyclase (Toda *et al.* 1985). Similarly, the SCF complex is essential for degradation of the Cdk1 inhibitor Sic1 at the G1/S transition (Schwob *et al.* 1994). However, the lethality of *cdc34-2* mutants as a consequence of low Ras signaling activity is not due solely to the fact that both Cdc34 and Ras are required for the G1/S transition. The viability of *cdc34-2 sic1*Δ mutants that are able to undergo a complete round of DNA replication is still dependent on glucose medium at elevated temperatures (Figure 7B).

The conclusion from these observations is that, under conditions where SCF activity is partially inactivated, cell viability depends on activated Ras signaling. This implies that either Ras activity accelerates the activity of the SCF directly or it bypasses the requirement of a fully active SCF for cell cycle progression. Proteolysis of Sic1 protein appears to be impaired severely in *cdc34-2* mutants incubated at semipermissive temperatures, and this effect was similar in *cdc34-2* and *cdc34-2 ras2*Δ cells (data not shown). Because *cdc34-2,* but not *cdc34-2 ras2*Δ, mutants are viable under these conditions, this implies that Ras2 signaling does not accelerate Sic1 proteolysis, but nevertheless restores cell viability to cells with low SCF activity. Proteolysis of other substrates of SCF may be influenced directly by Ras, or alternatively, high Ras activity may allow cell cycle progression despite partial defects in SCF activity. It remains unclear whether there is a direct or indirect effect of Ras activity on SCF function.

The findings that Ras activity influences both SCF and APC/C function suggest a role for Ras proteins in the coordination of the activities of these complexes during the cell cycle. At the G1/S transition, a switch occurs in APC/C- and SCF-mediated proteolysis of many cell-cycle-regulated proteins. The APC/C is turned off and, simultaneously, SCF-dependent proteolysis of proteins such as Sic1 and Cdc6 is activated (Amon *et al.* 1994; Schwob *et al.* 1994; Drury *et al.* 1997; Peters 1998). A second switch occurs in late mitosis when proteolysis of mitotic cyclins is activated at about the same time that Sic1 is starting to accumulate. Clearly, the main player causing these switches is Cdk1 activity, which phosphorylates Cdh1/Hct1 and Sic1 simultaneously. This prevents the association of Cdh1/Hct1 with the APC/C from S-phase to late anaphase and induces Sic1 instability during the same period (Verma *et al.* 1997; Zachariae *et al.* 1998a; Jaspersen *et al.* 1999). Ras activity might act in concert with Cdk1 to cause the switch from APC/C- to SCF-mediated proteolysis in late G1-phase. At the G1/S transition, activation of the Ras/cAMP pathway might accelerate SCF activity and at the same time reduce APC/C activity.

We further found that partial inactivation of the SCF complex, caused by *cdc4-1* and *cdc34-2* mutations, enhanced viability of *cdc23-1* and *cdc27-1* mutants defective in APC/C function. Thus, low SCF activity compensates for defects in the function of the APC/C, indicating that normal SCF activity has an inhibitory influence on the APC/C. However, it is unknown whether SCF affects APC/C function directly or indirectly. The SCF complex is needed to prevent Sic1 accumulation from late G1-phase until late anaphase, thereby ensuring high Cdk1 activity in this period of the cell cycle. In cells with a partially inactive SCF complex, Sic1 protein might accumulate abnormally, reduce Cdk1, and therefore enhance APC/C activity. Cdk1 is needed to keep the APC/C inactive during S- and G2-phase (Amon 1997) and a decrease in Cdk1 activity accelerates APC/C function (Figure 6). Thus, SCF function is crucial to keep Cdk1 highly active, thereby preventing abnormal APC/C activation and ensuring a proper regulation of its activity during the cell cycle. In conclusion, Ras signaling, Cdk1 activity, and SCF activity all have an inhibitory influence on the APC/C and therefore may be important for the regulation of this ubiquitin-ligase complex.

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