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Synergistic inhibition of APC/C by glucose and activated Ras proteins can be mediated by each of the Tpk1–3 proteins in *Saccharomyces cerevisiae*

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Proteolysis triggered by the anaphase-promoting complex/cyclosome (APC/C) is essential for the progression through mitosis. APC/C is a highly conserved ubiquitin ligase whose activity is regulated during the cell cycle by various factors, including spindle checkpoint components and protein kinases. The cAMP-dependent protein kinase (PKA) was identified as negative regulator of APC/C in yeast and mammalian cells. In the yeast *Saccharomyces cerevisiae*, PKA activity is induced upon glucose addition or by activated Ras proteins. This study shows that glucose and the activated $Ras2^{Val19}$ protein synergistically inhibit APC/C function via the cAMP/PKA pathway in yeast. Remarkably, Ras2 proteins defective in the interaction with adenylate cyclase fail to influence APC/C, implying that its function is regulated exclusively by PKA, but not by alternative Ras pathways. Furthermore, it is shown that the three PKAs in yeast, Tpk1, Tpk2 and Tpk3, have redundant functions in regulating APC/C in response to glucose medium. Single or double deletions of *TPK* genes did not prevent inhibition of APC/C, suggesting that each of the Tpk proteins can take over this function. However, Tpk2 seems to inhibit APC/C function more efficiently than Tpk1 and Tpk3. Finally, evidence is provided that Cdc20 is involved in APC/C regulation by the cAMP/PKA pathway.

INTRODUCTION

The anaphase-promoting complex/cyclosome (APC/C) is a highly conserved multisubunit complex which contains ubiquitin ligase activity (Peters, 2002; Zachariae & Nasmyth, 1999). APC/C is essential for two major events during mitosis: the separation of sister chromatids at the metaphase/anaphase transition and the exit from mitosis. The crucial role of APC/C in metaphase is ubiquitination of securins. In the budding yeast *Saccharomyces cerevisiae*, proteolytic destruction of securin Pds1 allows the separase Esp1 to become active (Amon, 2001; Nasmyth, 2002). Esp1 then cleaves the cohesin subunit Scc1 and thereby triggers sister chromatid separation. Important targets for the exit from mitosis are B-type cyclins, whose degradation leads to the inactivation of cyclin-dependent kinases (Morgan, 1999). Many other substrates of APC/C ubiquitin ligase have been identified, including the polo kinase Cdc5, kinesins, spindle-associated proteins and regulators of DNA replication (Harper *et al.*, 2002; Peters, 2002; Zachariae & Nasmyth, 1999).

APC/C activity is cell cycle regulated. It is kept inactive during S-, G2- and early M-phase, turned on during metaphase and then remains active throughout late M-phase and during the subsequent G1-phase. A variety of regulatory proteins of APC/C have been identified in the last few years. Most is known about the two proteins Cdc20 and Cdh1 (Peters, 2002). Recent data demonstrated that they function as substrate recognition proteins, which target substrates to the APC/C core complex (Hilioti *et al.*, 2001; Pfleger *et al.*, 2001; Schwab *et al.*, 2001; Vodermaier, 2001). An important feature is the temporal control of APC/C activation by Cdc20 and Cdh1 (Harper *et al.*, 2002; Peters, 2002). Cdc20 always precedes Cdh1 in binding and activation during mitosis. Both WD40 proteins have fundamental functions in controlling APC/C during mitosis. They are the targets of the spindle checkpoint, either directly or indirectly (Gardner & Burke, 2000). Factors of the spindle assembly checkpoint directly bind and inhibit Cdc20 in response to defects in the integrity of the mitotic spindle or in the bipolar attachment of kinetochores. A checkpoint monitoring the orientation of the mitotic spindle indirectly inhibits

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Abbreviations: APC/C, anaphase-promoting complex/cyclosome; MAPK, mitogen-activated protein kinase; PKA, protein kinase A.

the association of Cdh1 with APC/C and thereby delays cell division.

Further important regulatory proteins of APC/C are protein kinases, such as the cyclin-dependent kinase Cdk1 and polo kinase (Nigg, 2001). Both kinases were shown to trigger phosphorylation of specific APC/C subunits, known as Apc1, Cdc16, Cdc23 and Cdc27 (Golan *et al.*, 2002; Rudner & Murray, 2000). It was recently shown that either of these kinases is capable of activating APC/C, but both of them are required for efficient APC/C activation (Golan *et al.*, 2002).

A further protein kinase regulating APC/C activity is cAMP-dependent protein kinase (also termed protein kinase A or PKA). By using purified mammalian APC/C, it was shown that PKA directly phosphorylates the subunits Apc1 and Cdc27 *in vitro* (Kotani *et al.*, 1998). In contrast to Cdk1 and polo kinase, PKA-mediated phosphorylation inhibits APC/C activity. *In vitro* ubiquitination assays revealed that the addition of purified PKA blocked the ability of APC/C to catalyse the formation of polyubiquitin chains on cyclin B (Kotani *et al.*, 1999). These studies also showed that PKA may affect the binding of Cdc20 because Cdc20 failed to bind APC/C pre-incubated with PKA.

In budding and fission yeast, a direct phosphorylation of APC/C subunits by PKA has not yet been shown. Nevertheless, genetic data strongly implicated yeast PKA as negative regulator of APC/C, similar to the situation in mammalian cells. A variety of fission and budding yeast mutants defective in APC/C subunit genes were suppressed by reducing cAMP levels or PKA activity (Anghileri *et al.*, 1999; Irniger *et al.*, 2000; Yamada *et al.*, 1997; Yamashita *et al.*, 1996). It was shown that the addition of cAMP caused cell cycle arrest in mitosis, both at the metaphase/anaphase transition and in telophase (Anghileri *et al.*, 1999). Many yeast APC/C subunits contain multiple PKA consensus phosphorylation sequences (Kennelly & Krebs, 1991). The Apc1 subunit for example contains 28 of these motifs.

In budding yeast, the cAMP/PKA pathway can be activated either by the addition of glucose to cells grown on poor carbon sources or by the activation of Ras proteins (Broach, 1991; Thevelein & de Winde, 1999). Recent data showed that the glucose signal is not transmitted by Ras proteins to adenylate cyclase Cyr1, as previously thought. Instead, a G-protein-receptor system, consisting of the receptor Gpr1 and the Gα protein Gpa2, stimulates adenylate cyclase in response to glucose addition (Colombo *et al.*, 1998).

Activation of the small GTP-binding proteins Ras1 and Ras2 is catalysed by Cdc25, a protein that promotes the exchange of GDP with GTP on Ras (Broek *et al.*, 1987; Robinson *et al.*, 1987). Ras-GTP stimulates adenylate cyclase and thereby induces an increase in cAMP levels and activation of PKA (Toda *et al.*, 1985). In budding yeast, this kinase is encoded by three separate genes, *TPK1*, *TPK2* and *TPK3* (Toda *et al.*, 1987). In the presence of cAMP the inhibitory protein Bcy1 releases the catalytic subunits which are then able to phosphorylate their target proteins (Broach, 1991; Thevelein & de Winde, 1999).

Consistent with the findings that PKA negatively regulates APC/C, we have previously shown that glucose medium and activation of Ras signalling is lethal for mutants defective in APC/C function (Irniger *et al.*, 2000). Mutations in APC/C subunit genes, such as *apc10-22* or *cdc27-1*, were suppressed either by decreasing Ras activity or by growth on the poor carbon source raffinose. In contrast, a constitutively activated $RAS2^{Val19}$ allele or shifts to glucose medium were deleterious to these mutants. In this study, we show that glucose and activated $Ras2^{Val19}$ synergistically cause APC/C inhibition and that Tpk1, Tpk2 and Tpk3 apparently have overlapping functions in this process.

METHODS

Yeast strains and plasmids. Yeast strains used in this study are derivatives of the *Saccharomyces cerevisiae* W303 strain (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3 GAL psi+*) and are listed in Table 1. All strains obtained from a different background were backcrossed at least four times to W303 strains to make them congenic. Plasmids containing the $RAS2^{Val19}$, $RAS2^{Val19Gly41}$ and $RAS2^{Val19Asn45}$ alleles on the centromeric plasmid YCplac33 (Mösch *et al.*, 1999), the *TPK1*, *TPK2* and *TPK3* genes on the high-copy plasmid pRS426 (Mösch *et al.*, 1999) and the *STE11-4* allele

Table 1. Yeast strains used in this study

Strain	Relevant genotype
S001	Wild-type strain W303 *MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL psi+*
S095	*MAT***a** *apc10-22*
S201	*MATα cdh1::HIS5*
S211	*MAT***a** *apc10-22 cdh1::HIS5*
S221	*MAT***a** *cdc27-1*
S320	*MAT***a** *apc10-22 tpk1*::Kan-R
S321	*MAT***a** *apc10-22 tpk2*::Kan-R
S322	*MAT***a** *tpk1*::Kan-R
S323	*MAT***a** *tpk2*::Kan-R
S324	*MAT***a** *cdc27-1 tpk2*::Kan-R
S325	*MAT***a** *apc10-22 tpk3*::Kan-R
S326	*MAT***a** *cdc27-1 tpk3*::Kan-R
S327	*MAT***a** *tpk3*::Kan-R
S328	*MAT***a** *cdc27-1 tpk1*::Kan-R
S330	*MAT***a** *tpk2*::*tpk1*::Kan-R
S331	*MAT***a** *tpk2*::*tpk3*::Kan-R
S332	*MAT***a** *tpk3*::*tpk1*::Kan-R
S333	*MAT***a** *cdc27-1 tpk2*::*tpk1*::Kan-R
S334	*MAT***a** *cdc27-1 tpk2*::*tpk3*::Kan-R
S335	*MAT***a** *cdc27-1 tpk3*::*tpk1*::Kan-R
S336	*MAT***a** *apc10-22 tpk2*::*tpk1*::Kan-R
S337	*MAT***a** *apc10-22 tpk2*::*tpk3*::Kan-R
S444	*MAT***a** *apc10-22 tpk1*::*tpk3*::Kan-R
S469	*MAT***a** *GAL–HA3–CDH1::TRP1*
S481	*MAT***a** *MYC18–CDC20::TRP1*

(Stevenson *et al.*, 1992) cloned into the centromeric plasmid YCp50 were previously described. *TPK* genes expressed from the inducible *GAL1* promoter were constructed by PCR amplification of the corresponding genes and subsequent fusions to the *GAL1* promoter on the 2μ plasmid YEplac195. The *GAL–CDC20* gene fusion was isolated from a *GAL* cDNA library (unpublished results).

For deletion of a single *TPK* gene (*TPK1*, *TPK2* or *TPK3*), a deletion cassette containing the kanamycin resistance gene, *kan-R*, from the *Escherichia coli* transposon Tn*903* flanked by the 5′-region and the 3′-region of the *TPK* gene, was transformed into yeast strains. Transformants were selected on YEPD + Geneticin plates and restreaked on YEPD + Geneticin plates. Deletions in wild-type, *apc10-22* and *cdc27-1* strains were verified by Southern hybridization.

Double deletions of *TPK* genes were constructed starting from the single deletion strains. The *tpk*-deletion cassette contains on both sides of the *kan-R* marker gene a *loxP* recombination sequence from the bacteriophage P1 (Güldener *et al.*, 1996). By recombination of both *loxP* sequences, the *kan-R* sequence was removed from the genome and the strain regained sensitivity to Geneticin. Afterwards another *tpk* deletion cassette was transformed into the single deletion strain and the *kan-R* gene served as selection marker for the second *tpk* deletion. Plasmid pSH47 (Güldener *et al.*, 1996) containing the Cre recombinase under control of the inducible *GAL1* promoter and a *URA3* selection marker was transformed in a first step into *tpk* single deletion strains. Induction of the *GAL1* promoter resulted in the expression of the Cre recombinase, which performed recombination of the *loxP* sequences. Loss of the *kan-R* marker gene was verified by selection for Geneticin-sensitive transformants.

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

When yeast cells were grown in complete medium, YEP medium (2 % bactopeptone, 1 % yeast extract, 0·005 % adenine sulfate) supplemented with 2 % glucose (YEPD), 2 % galactose (YEP + Gal) or 2 % raffinose (YEP + Raf) was used. For the selection of plasmid-containing strains, cells were grown in minimal medium, a synthetic medium containing 0·8 % yeast nitrogen base and 50 μg ml^{-1} each of uracil and adenine, supplemented with amino acids and 2 % glucose or 2 % raffinose (Rose *et al.*, 1990).

Growth conditions and cell cycle arrest. Prior to the incubation of mutant strains at elevated temperature on agar plates, the cells were always pre-incubated at 25 °C for 12–18 h. Prior to cell cycle arrest in liquid medium, cultures were pre-grown to OD_{600} 0·3–0·6 at 25 °C. When a gene was expressed from the inducible *GAL1* promoter, cells were pre-grown in medium containing raffinose as the sole carbon source. The *GAL1-10* promoter was induced by the addition of 2 % galactose. To arrest cells in G1 phase with α-factor pheromone (Nova Biochem), 5 μg α-factor ml^{-1} was added. For prolonged α-factor treatments, additional α-factor was added after every 120 min to prevent a drop in the α-factor concentration.

Immunoblotting. 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. MYC and HA antibodies were both used in 1 : 100 dilutions.

RESULTS

Glucose and activated Ras2 proteins synergistically inhibit APC/C function

In previous experiments we showed that glucose or activated Ras2 proteins are deleterious for temperature-sensitive mutants in APC/C subunit genes (Irniger *et al.*, 2000). Since it was reported earlier that glucose activates adenylate cyclase independently of Ras proteins (Colombo *et al.*, 1998; Thevelein & de Winde, 1999), we tested whether this model may also apply to the regulation of APC/C. We argued that if the glucose signal and Ras proteins acted via separate pathways on adenylate cyclase, then the simultaneous activation of both pathways may have a synergistic effect on adenylate cyclase, PKA activity and inhibition of APC/C.

To test this model, *apc10-22* mutants were transformed with a centromeric plasmid containing either the constitutively activated $RAS2^{Val19}$ allele or, as control, the empty vector YCplac22 (*TRP1* marker). Transformants were pregrown at 25 °C in minimal medium lacking tryptophan (−Trp) and containing raffinose as sole carbon source. Then cells were streaked to fresh −Trp plates containing either glucose or raffinose. *apc10-22* mutants containing $RAS2^{Val19}$ were viable at 28 °C on raffinose plates, but non-viable on glucose plates, displaying severe growth defects even at 25 °C (Fig. 1). *apc10-22* cells carrying the control plasmid were viable under these conditions. *apc10-22* $RAS2^{Val19}$ cells were non-viable on raffinose plates at 34 °C, a temperature tolerated by *apc10-22* mutants carrying the control plasmid. Both strains were non-viable on glucose medium at this temperature. These findings show that both glucose and activated Ras proteins interfere with viability of *apc10-22* mutants. The lethality of *apc10-22* $RAS2^{Val19}$ cells at 28 °C demonstrates that glucose and the activated Ras2 protein synergistically reduce the viability of *apc10-22* mutants. Similar findings were observed for *cdc27-1* mutants (data not shown). Thus, glucose and activated Ras2 have a combined effect on APC/C function.

Inhibition of APC/C by activated Ras2 requires the PKA pathway

We next asked whether activated Ras proteins mediate their effect on APC/C by cAMP/PKA or whether other pathways may be involved. Ras1 and Ras2 activate two different signalling pathways, the cAMP/PKA pathway and the mitogen-activated protein kinase (MAPK) pathway consisting of Cdc42/Ste20/Ste11/Ste7/Kss1/Ste12 proteins (Mösch, 2000). For invasive growth of yeast, activation of either of these pathways by Ras was sufficient, indicating that cAMP/PKA and MAPK signalling had redundant functions in transmitting the signal from Ras to the effectors (Mösch *et al.*, 1999).

To test whether APC/C inhibition by activated Ras may also be transmitted by both of these pathways, we used *RAS2* alleles which contained, in addition to the activating

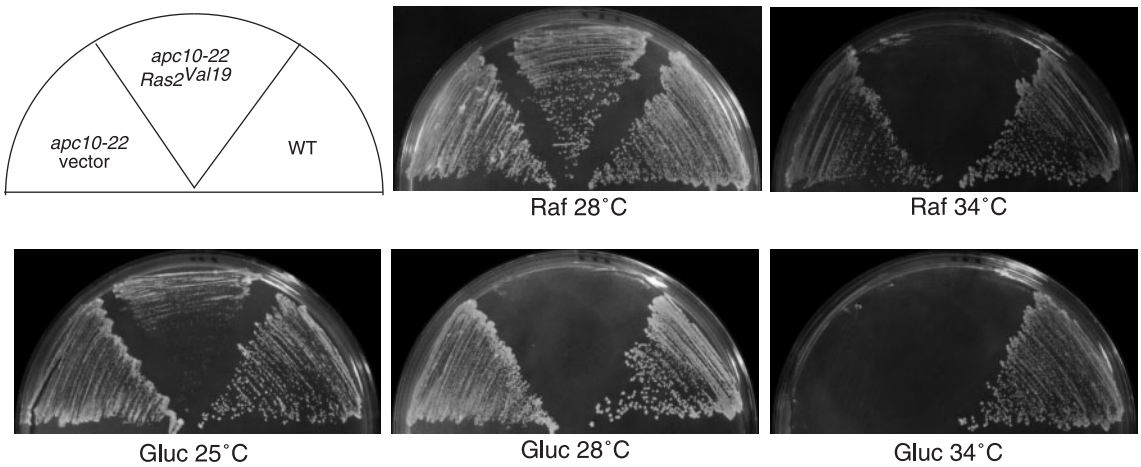


Fig. 1. Glucose and the activated *RAS2*^{Val19} allele have a combined lethal effect on *apc10-22* mutants. *apc10-22* mutants carrying either a centromeric plasmid with the *RAS2*^{Val19} gene or an empty vector plasmid (YCplac22, containing the *TRP1* marker) were pregrown on minimal medium lacking tryptophan and containing raffinose as carbon source (MM−Trp+Raf). Cells were then streaked on MM−Trp plates containing either raffinose or glucose and incubated for 2·5 days at the indicated temperatures. A wild-type strain was used as control.

mutation in the Val19 codon, second-site mutations at codons 41 and 45. Exchanges of Pro41 to Gly and Asp45 to Asn were shown to cause defects in the binding and activation of adenylate cyclase, but these proteins were still able to activate the MAPK pathway (Mösch *et al.*, 1999). Thus, Ras2^{Val19} is able to activate both pathways, but Ras2^{Val19Gly41} and Ras2^{Val19Asn45} functions are restricted to the MAPK pathway. Centromeric plasmids containing *RAS2*^{Val19}, *RAS2*^{Val19Gly41} or *RAS2*^{Val19Asn45} genes, or no insert, were transformed into a wild-type strain and into the *apc* mutant strains *apc10-22* and *cdc27-1*. The plasmid-carrying strains were grown at 34 °C or 30 °C, respectively, on −Trp minimal medium with raffinose as the sole carbon source. In contrast to mutant cells carrying *RAS2*^{Val19}, *apc10-22* and *cdc27-1* transformants with the double mutations in the *RAS2* genes were viable under these conditions (Fig. 2a, b). *apc10-22* mutants containing *RAS2*^{Val19Gly41} or *RAS2*^{Val19Asn45} were also viable on glucose medium at 28 °C and therefore do not display the synergistic phenotype observed when glucose and activated Ras2^{Val19} protein were combined (Fig. 2c). We conclude that Ras2 proteins which are defective in binding adenylate cyclase do not affect APC/C function. Thus, the signal from Ras appears to be transmitted through cAMP and PKA. In contrast to signalling for the induction of invasive growth, the MAPK pathway is apparently unable to replace the cAMP/PKA pathway.

Activation of the MAPK pathway does not influence *apc* mutants

To further elucidate whether the MAPK pathway may cause APC/C inhibition, we transformed *apc10-22* mutants with plasmids carrying a hyperactive *STE11* allele, *STE11-4* (Stevenson *et al.*, 1992), on the centromeric plasmid YCp50. The Ste11 protein kinase is one component of the MAPK signalling cascade. We argued that APC/C inhibition caused by highly active MAPK signalling would interfere with viability of *apc* mutants, as seen for Ras2^{Val19}. Transformants carrying plasmids with either the *STE11-4* allele or no insert were tested on minimal medium containing raffinose as the sole carbon source. We found that *apc10-22* containing *STE11-4* produced colonies on raffinose plates, similar to mutant strains with the empty vector (Fig. 2d). Mutant cells containing either of the plasmids were non-viable on glucose plates. Thus, APC/C is apparently neither positively nor negatively affected by activation of the MAPK cascade.

These results support the model that APC/C inhibition caused by activated Ras signalling is mediated exclusively by the PKA pathway and not by the MAPK pathway.

Each of the Tpk1–3 proteins is sufficient for APC/C inhibition in response to glucose

Previous findings have shown that for some of the functions of yeast PKA, the Tpk1, Tpk2 and Tpk3 proteins are redundant, whereas other processes, such as the regulation of pseudohyphal growth, require one specific Tpk protein (Robertson *et al.*, 2000; Robertson & Fink, 1998). To test the role of the three Tpk proteins in APC/C inhibition, we constructed *apc10-22* and *cdc27-1* mutants containing either single deletions or double deletions of *TPK* genes. We argued that if APC/C inhibition were mediated by a specific Tpk protein then a deletion of the corresponding gene would abolish lethality of *apc* mutations upon shift to glucose medium.

A *cdc27-1* strain and *cdc27-1* strains containing single *tpk* deletions (*cdc27-1 tpk1*Δ, *cdc27-1 tpk2*Δ, *cdc27-1 tpk3*Δ) or double *tpk* deletions (*cdc27-1 tpk1*Δ *tpk2*Δ, *cdc27-1 tpk1*Δ

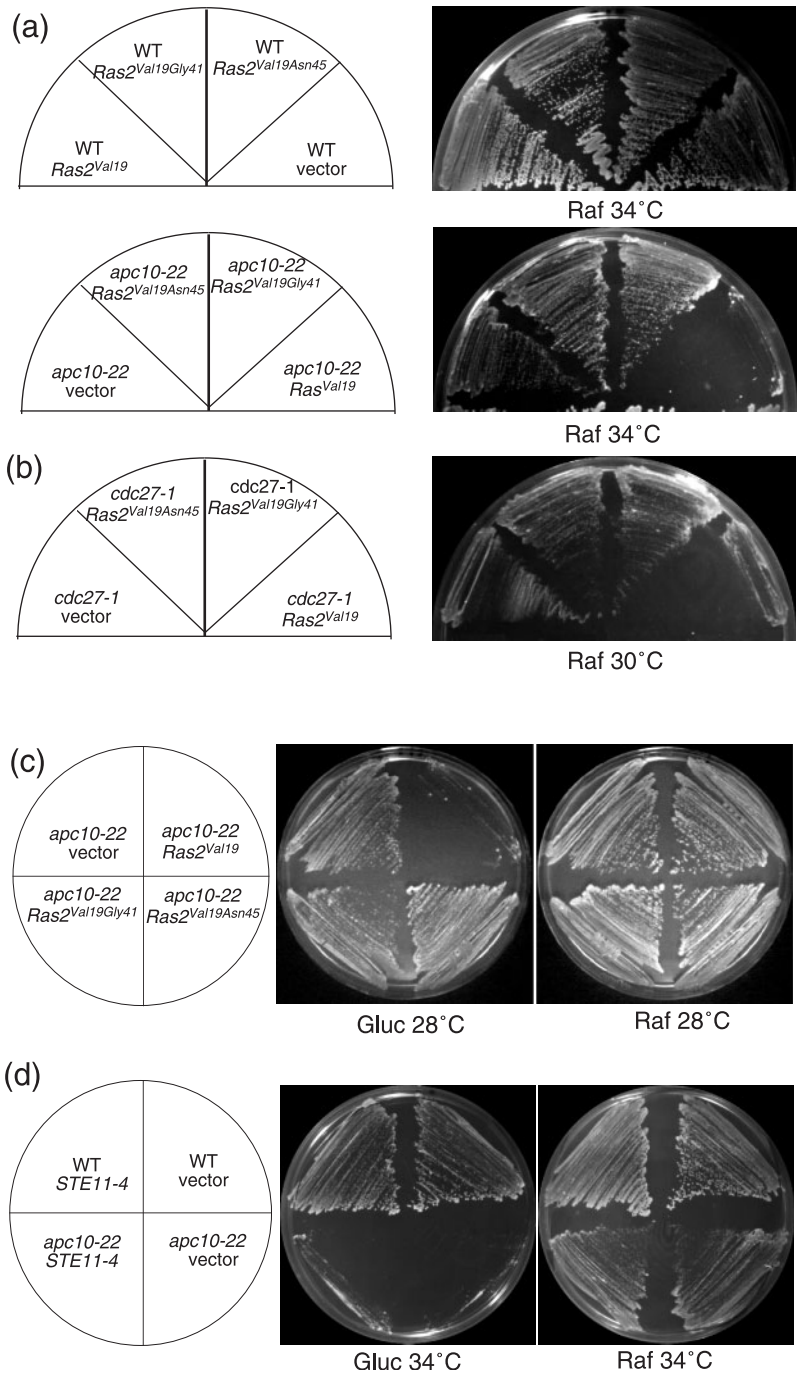


Fig. 2. Activated *RAS2* alleles defective in stimulating adenylate cyclase are not lethal for *apc10-22* and *cdc27-1* mutants. (a, b) Centromeric plasmids carrying either the $RAS2^{Val19}$, $RAS2^{Val19Gly41}$ or $RAS2^{Val19Asn45}$ gene, or the empty plasmid YCplac22 (containing *TRP1* as selectable marker), were transformed into a wild-type strain and the *apc10-22* and *cdc27-1* mutant strains. Transformants were pregrown on MM−Trp+Raf medium at 25 °C, then streaked onto fresh plates and shifted to semi-permissive temperatures: 34 °C for *apc10-22* mutants (a) and 30 °C for *cdc27-1* mutants (b). Plates were incubated for 2·5 days. (c) Transformants of *apc10-22* mutants were streaked onto MM−Trp plates containing either glucose or raffinose and incubated at 28 °C for 2·5 days. (d) Wild-type and *apc10-22* mutants were transformed with centromeric plasmids containing the *STE11-4* allele or the empty plasmid YCplac50 (with *URA3* as selectable marker). Transformants were pregrown on MM−Ura+Raf at 25 °C, streaked onto fresh plates containing either glucose or raffinose and incubated at 34 °C for 2·5 days.

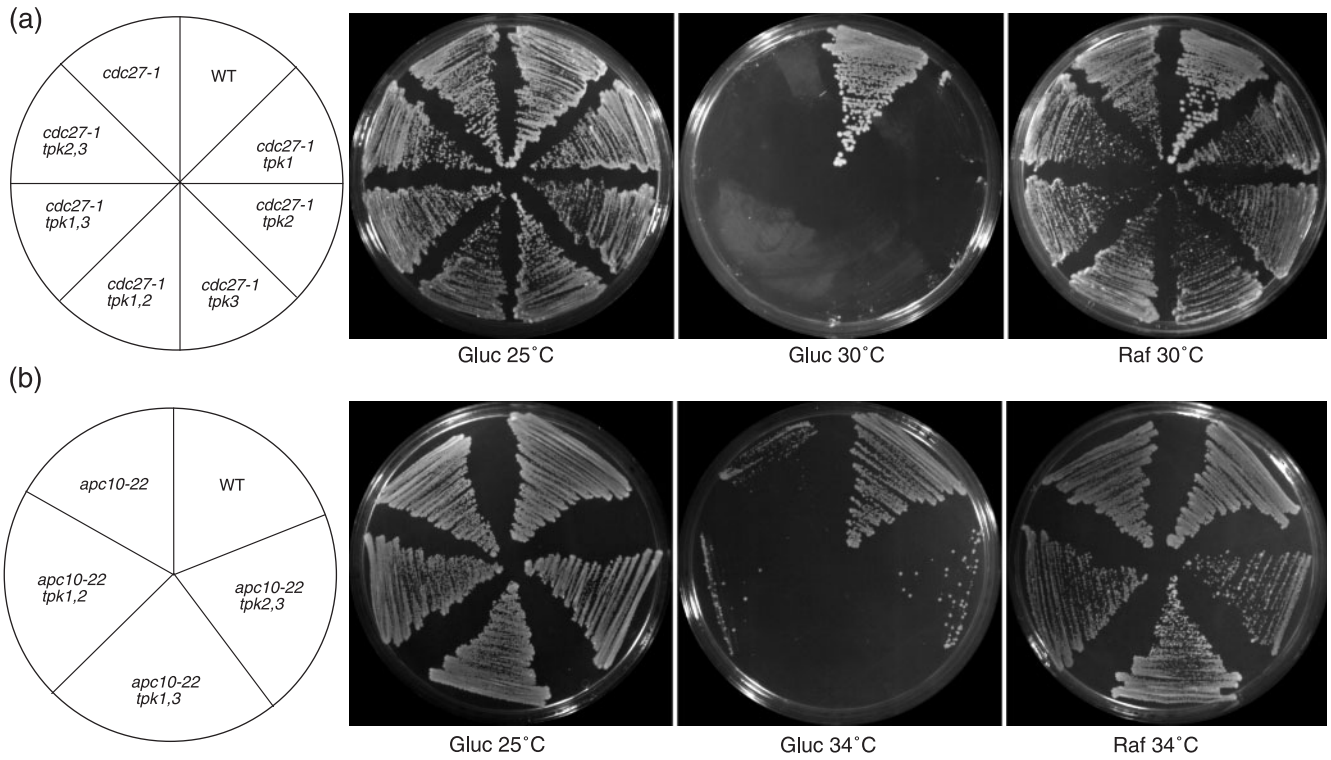
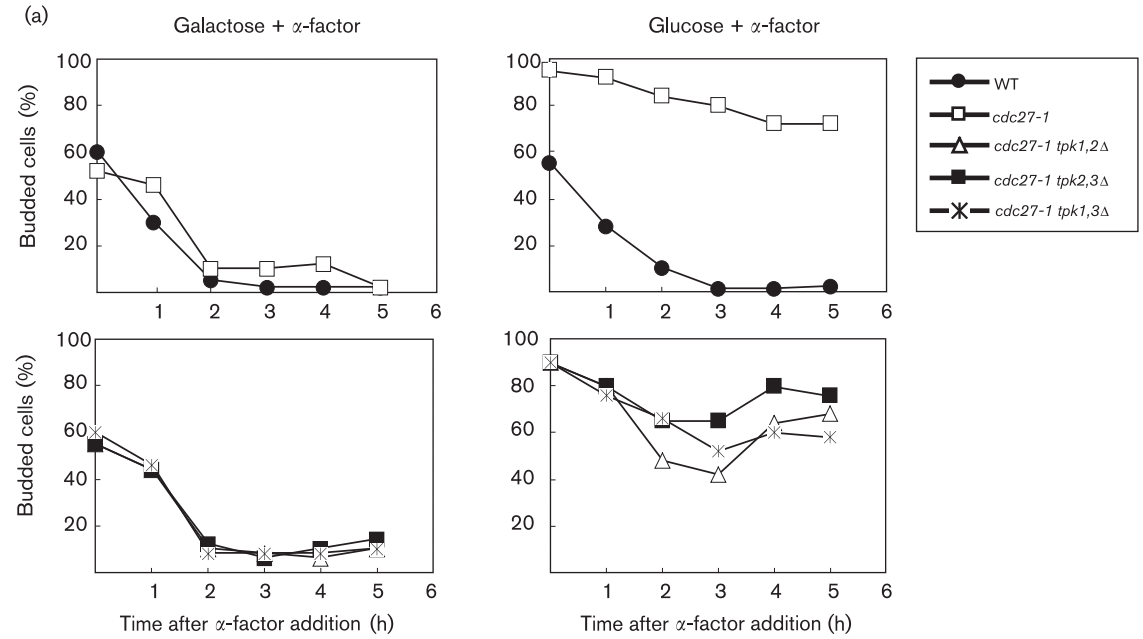


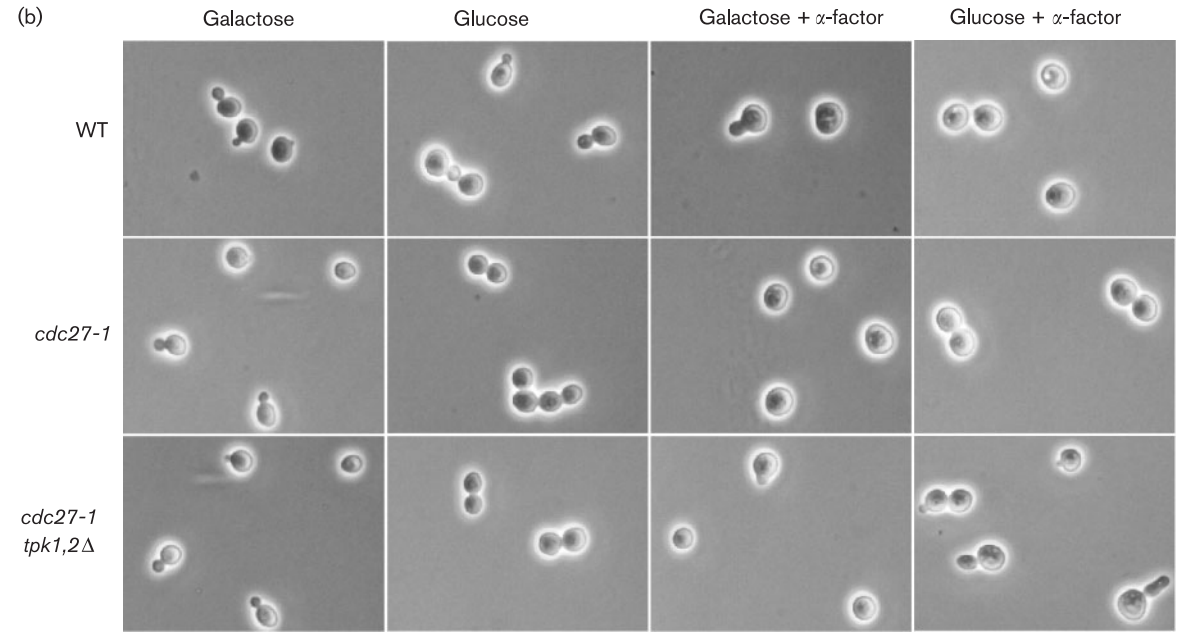
Fig. 3. One Tpk protein is sufficient for APC/C inhibition in response to glucose. (a) *cdc27-1* mutant strains deleted for a single *TPK* gene (*cdc27-1 tpk1*Δ, *cdc27-1 tpk2*Δ, *cdc27-1 tpk3*Δ) or deleted for two *TPK* genes (*cdc27-1 tpk1*Δ *tpk2*Δ, *cdc27-1 tpk1*Δ *tpk3*Δ, *cdc27-1 tpk2*Δ *tpk3*Δ) were pregrown on YEP + Raf plates at 25 °C, then streaked to fresh YEP + Glu or YEP + Raf plates and incubated at either 25 °C or 30 °C for 2·5 days. (b) *apc10-22* mutants deleted for two *TPK* genes (*apc10-22 tpk1*Δ *tpk2*Δ, *apc10-22 tpk1*Δ *tpk3*Δ, *apc10-22 tpk2*Δ *tpk3*Δ) were treated as in (a), but incubated at either 25 °C or 34 °C.

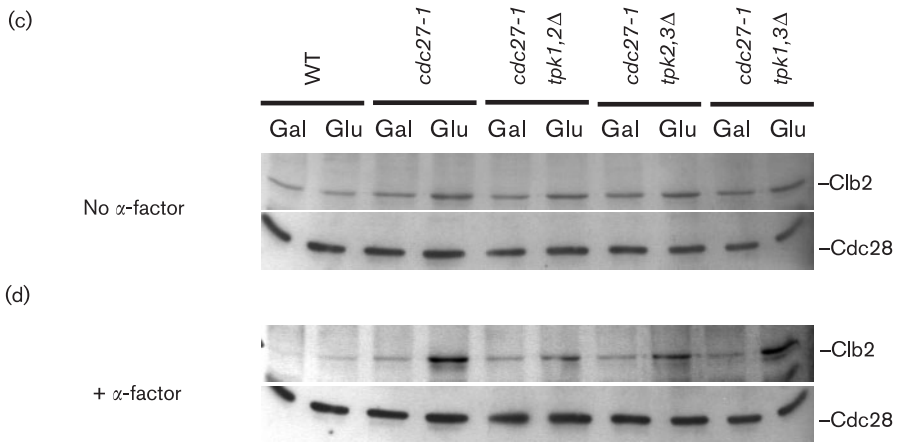
*tpk3*Δ, *cdc27-1 tpk2*Δ *tpk3*Δ) were incubated on YEP plates containing either glucose or raffinose at 30 °C, a semi-permissive temperature for *cdc27-1* mutants. All *cdc27-1* strains were viable at 30 °C on raffinose medium, but failed to form colonies in the presence of glucose (Fig. 3a). Similarly, an *apc10-22* mutant and each of the derivative *tpk* double deletions grew on raffinose medium, but were non-viable on YEPD at the semi-permissive temperature, 34 °C (Fig. 3b). These results show that single or double deletions of *TPK* genes do not affect the viabilty of *apc* mutants. Thus, it appears that each of the *TPK* genes is sufficient for signal transmission from glucose to APC/C, suggesting that the three *TPK* genes fulfil a redundant function in this process.

To further test the effect of glucose on *apc* mutants containing only a single *TPK* gene, *cdc27-1* and *cdc27-1 tpk* double deletion strains were first pregrown in raffinose medium at 28 °C. Under these conditions, *cdc27-1* mutants undergo cell cycle progression without obvious defects when using raffinose as carbon source. Cultures were split in two halves and either glucose or, as control, the poor carbon source galactose were added. We argued that glucose-induced inhibition of APC/C function would block *cdc27-1* cells in mitosis. To monitor whether glucose causes *cdc27-1* mutants to arrest in mitosis, α-factor was added and the cultures were further incubated at 28 °C. α-Factor induces cells to arrest as unbudded cells in G1-phase. We found that *cdc27-1* as well as *cdc27-1* strains containing only one *TPK* gene were blocked in mitosis upon glucose addition (Fig. 4a, b). After 3 h incubation in the presence of pheromone,

Fig. 4. One Tpk protein is sufficient for the mitotic arrest of *cdc27-1* mutants. A wild-type strain, a *cdc27-1* mutant strain and *cdc27-1* mutants deleted for two *TPK* genes (*cdc27-1 tpk1*Δ *tpk2*Δ, *cdc27-1 tpk1*Δ *tpk3*Δ, *cdc27-1 tpk2*Δ *tpk3*Δ) were pregrown on YEP + Raf medium at 28 °C. Then, either 2 % glucose or 2 % galactose was added and cells were further incubated for 5 h at the same temperature. Subsequently, α-factor was added to arrest cells, which are able to complete mitosis, in G1 phase. (a) Percentage of budded cells at the indicated time points after α-factor addition. Values are means of three independent experiments. (b) Photographs of cells of the indicated strains grown on galactose or glucose medium before α-factor addition (left) and 5 h after α-factor addition (right). (c, d) Immunoblotting of Clb2 protein levels before (c) or after (d) α-factor addition using Clb2 antibodies. Cdc28 was used as loading control.


(a)
Galactose + α-factor
Glucose + α-factor
Budded cells (%)
Time after α-factor addition (h)
WT
cdc27-1
cdc27-1 tpk1,2Δ
cdc27-1 tpk2,3Δ
cdc27-1 tpk1,3Δ


(b)
Galactose
Glucose
Galactose + α-factor
Glucose + α-factor
WT
cdc27-1
cdc27-1 tpk1,2Δ


(c)
WT
cdc27-1
cdc27-1 tpk1,2Δ
cdc27-1 tpk2,3Δ
cdc27-1 tpk1,3Δ
Gal Glu Gal Glu Gal Glu Gal Glu Gal Glu
No α-factor
–Clb2
–Cdc28

(d)
+ α-factor
–Clb2
–Cdc28

40–70 % of cells were still large-budded. In contrast, most cells arrested in G1-phase as unbudded cells in the cultures treated with galactose, similar to wild-type cells. In these cultures, the proportion of budded cells rapidly decreased after α-factor addition. Importantly, each of the *cdc27-1 tpk* double deletion strains shows a similar phenotype to the *cdc27-1* strain upon glucose or galactose addition.

To test whether indeed APC/C-mediated proteolysis is impaired in the *cdc27-1 tpk* double deletion strains upon treatment with glucose, we determined protein levels of the mitotic cyclin Clb2, a substrate of APC/C. Clb2 levels were analysed by immunoblotting before and 3 h after pheromone treatment. Before α-factor addition, Clb2 was present in all cultures, but Clb2 protein levels were marginally higher in *cdc27-1* mutant strains treated with glucose (Fig. 4c). After α-factor addition Clb2 levels dropped to low levels in wild-type cells and in *cdc27-1* cells incubated in galactose medium. In contrast, cyclin levels remained high in *cdc27-1* cells grown in glucose medium (Fig. 4d). In *cdc27-1 tpk1*Δ *tpk3*Δ cells, Clb2 levels were similar to those in *cdc27-1* cells, but they were partially decreased in the other double *tpk* deletion strains. These results indicate that Tpk2 has a more potent function than Tpk1 and Tpk3 in APC/C regulation. However, Clb2 degradation was at least partially affected in each *cdc27-1* strain with double *tpk* deletions in the presence of glucose. Thus, the inhibitory signal from glucose to APC/C is transmitted in each of these strains.

Taken together, these results show that one single Tpk protein is sufficient for APC/C inhibition in response to glucose and that Tpk2 may be particularly efficient in this process.

Overexpression of *TPK* genes is deleterious to *apc* mutants

To further test whether Tpk proteins may mediate APC/C inhibition with different efficiencies, we determined the effects of high levels of Tpk proteins on the viability of *apc* mutants. *cdc27-1* and *apc10-22* mutants were transformed with high-copy plasmids containing the *TPK1*, *TPK2* or *TPK3* gene. The viability of these mutants at elevated temperatures was determined on −Ura minimal medium containing raffinose (Fig. 5). Consistent with our data suggesting that Tpk2 efficiently inhibits APC/C (Fig. 4d), we found that overexpression of *TPK2* caused a distinct reduction of the viability of *apc* mutants. In contrast, the *TPK1*-containing plasmid only marginally affected *apc* mutants. Remarkably, overexpressed *TPK3* also efficiently interferes with the viability of these mutants. Previously, Tpk3 was shown to have a low catalytic activity, but this was apparently due to the poor expression of the *TPK3* gene (Mazon *et al.*, 1993). When present in high levels, Tpk3 also appears to have high catalytic activity and thereby efficiently inhibits APC/C function. Since *TPK3* is only expressed to low levels in cells containing single copies of *TPK* genes, Tpk2 is apparently the most efficient Tpk protein in mediating APC/C inhibition in response to activation of the cAMP/PKA signalling pathway.

Glucose affects APC/C independently of the regulatory protein Cdh1

Cdh1 and Cdc20 are regulatory proteins of APC/C and we addressed the question whether the cAMP/PKA pathway affects APC/C function by regulating these proteins. To find out whether PKA regulates APC/C activity via Cdh1, we first

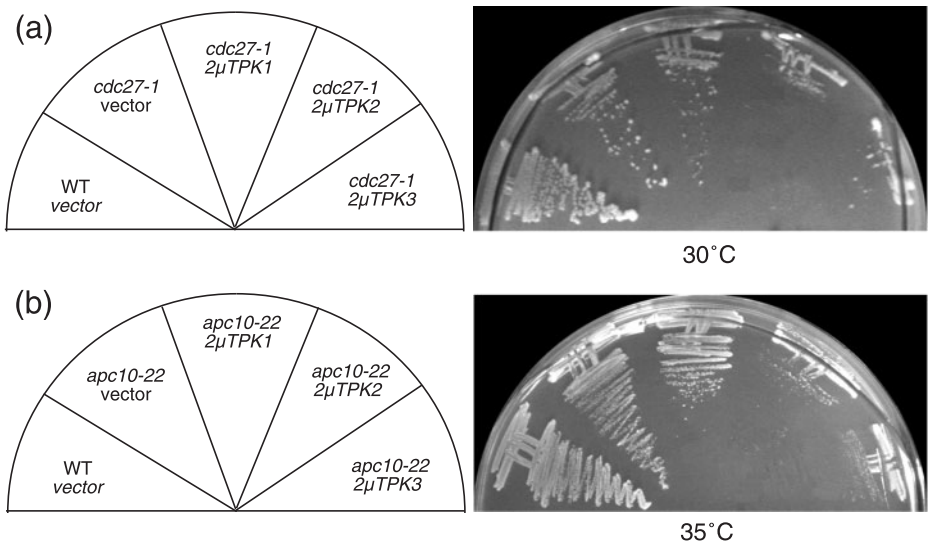


Fig. 5. Different effects of overexpressing different *TPK* genes on *cdc27-1* and *apc10-22* mutants. *cdc27-1* and *apc10-22* mutants carrying either *TPK1*, *TPK2*, *TPK3* or no insert in a high-copy plasmid (with *URA3* as selectable marker) were streaked to minimal medium lacking uracil and containing raffinose as carbon source. Plates were incubated at (a) 30 °C or (b) 35 °C for 2·5 days. A wild-type strain was used as control.

tested the ability of PKA to phosphorylate Cdh1. Cdh1 was previously shown to be phosphorylated by Cdk1 and this modification can be monitored by mobility shifts in immunoblots (Zachariae *et al.*, 1998). In G1 cells, Cdk1 is inactive and Cdh1 is not phosphorylated. This allowed us to test whether Tpk proteins are able to trigger Cdh1 phosphorylation. We induced the expression of *TPK1*, *TPK2* or *TPK3* genes in α-factor-arrested G1 cells and analysed the mobility of Cdh1 by immunoblotting. In contrast to cycling cells, no slower-migrating forms of Cdh1 were detectable upon the expression of *TPK* genes to high levels, indicating that Cdh1 is not a target of Tpk proteins (Fig. 6a).

We tested the possible role of Cdh1 in PKA-mediated APC/C regulation by an alternative experiment, by constructing *apc10-22* mutants lacking the non-essential *CDH1* gene. We argued that if PKA regulates APC/C activity predominantly by Cdh1 phosphorylation, then a deletion of *CDH1* would abolish the lethal effect of glucose on *apc10-22* mutants. *apc10-22 cdh1*Δ mutants were tested with regard to their viability on glucose and raffinose medium. On glucose medium, *apc10-22* and the double mutant *apc10-22 cdh1*Δ were unable to grow at 34 °C (Fig. 6b). Both strains were viable on YEP + Raf medium at this temperature. Thus, the absence of the regulatory protein Cdh1 has no influence on the viability of *apc10-22* mutants on glucose or raffinose

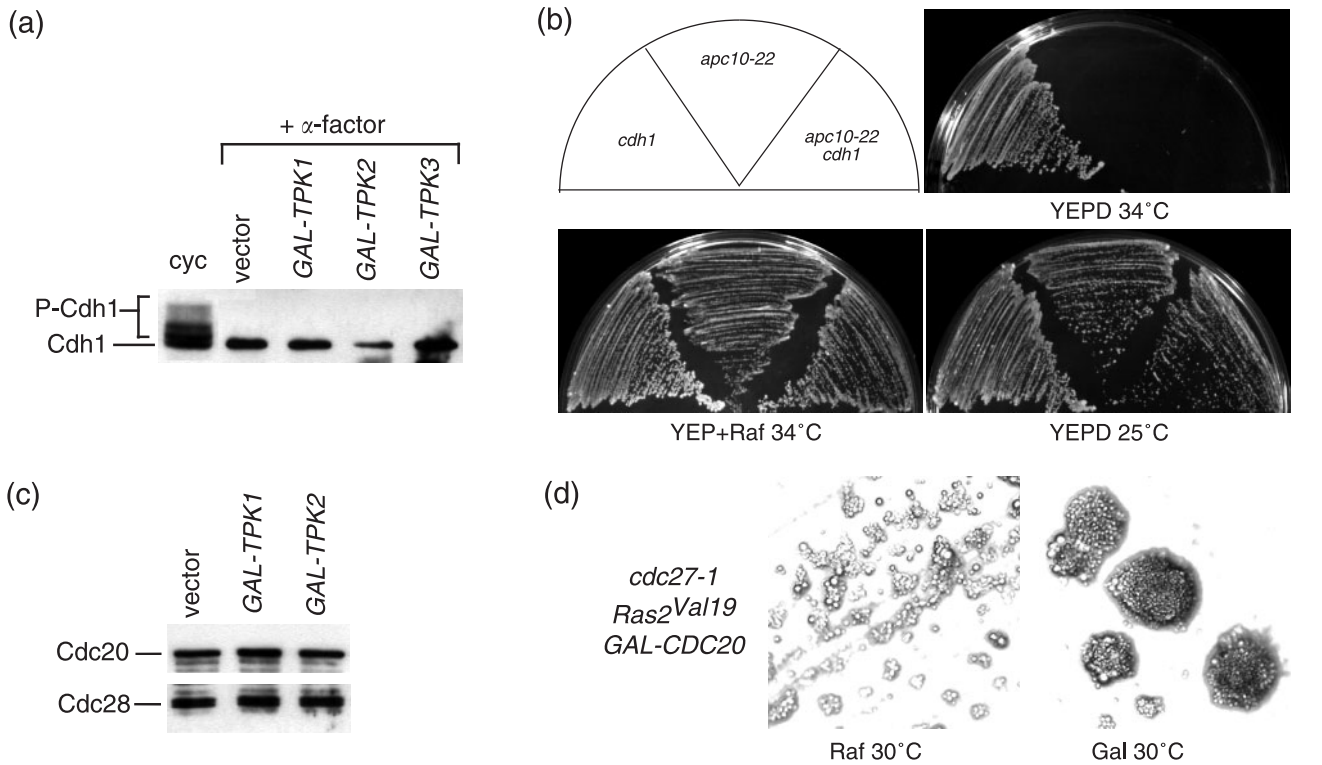


Fig. 6. APC/C inhibition in response to glucose occurs independently of Cdh1, but may involve Cdc20. (a) Strains containing a *GALL–HA3–CDH1* construct and carrying either a *GAL–TPK1*, *GAL–TPK2* or *GAL–TPK3* fusion or no insert on a 2μ plasmid (with *URA3* as selectable marker) were pregrown in MM−Ura + Raf medium. α-Factor was added to arrest cells in G1 phase. Then galactose was added to induce expression of the corresponding fusion genes and cells were incubated for 2 h. Cdh1 protein was analysed by immunoblotting using the HA antibody. A cycling culture was used as control for the detection of phosphorylated Cdh1. The faster-migrating additional bands visible in lanes 4 and 5 are most likely degradation products. (b) *cdh1*Δ, *apc10-22* and *apc10-22 cdh1*Δ strains were pregrown on YEP + Raf plates at 25 °C, streaked onto fresh YEP + Raf or YEP + Glu (YEPD) plates and then incubated at either 25 °C or 34 °C for 2·5 days. (c) A strain containing an N-terminally Myc18-tagged *CDC20* gene and either a *GAL–TPK1* or a *GAL–TPK2* fusion or no insert on a 2μ plasmid (with *URA3* as selectable marker) were pregrown in MM−Ura + Raf medium. Nocodazole was added to arrest cells in mitosis. Then galactose was added to express the corresponding fusion genes and cells were incubated for 2 h. Cdc20 protein was analysed by immunoblotting using the MYC antibody. (d) A *cdc27-1* mutant carrying a centromeric plasmid containing a *GAL–CDC20* fusion (with *URA3* as selectable marker) and a centromeric plasmid containing *RAS2*^{Val19} (with *TRP1* as selectable marker) were pregrown on minimal medium lacking uracil and tryptophan and containing raffinose at 30 °C. Cells were then streaked onto minimal medium lacking uracil and tryptophan and containing raffinose and galactose to induce *CDC20* expression. Cells were incubated at 30 °C and photographed after 40 h incubation.

medium. These results indicate that inhibition of APC/C activity in response to glucose occurs independently of Cdh1.

Evidence for Cdc20 as potential target of the cAMP/PKA pathway

We next aimed to determine whether cAMP/PKA-mediated inhibition of APC/C function may involve the Cdc20 protein. Cdc20 protein levels are cell cycle regulated by transcriptional and post-transcriptional mechanisms (Harper *et al.*, 2002). We first tested whether high PKA activity affects Cdc20 protein levels. A yeast strain containing an N-terminally Myc-tagged Cdc20 (Myc18–Cdc20; Shirayama *et al.*, 1998) was transformed with high-copy plasmids containing either *GAL–TPK1* or *GAL–TPK2* fusions. Cells were then grown in raffinose medium and arrested in metaphase with the microtubule-depolymerizing drug nocodazole. In this period of the cell cycle, Cdc20 protein levels are normally high (Shirayama *et al.*, 1998). To determine whether the overexpression of *TPK1* or *TPK2* genes affects Cdc20 protein levels, galactose was added and Cdc20 was analysed by immunoblotting (Fig. 6c). We found that Cdc20 protein levels remained the same under conditions of low or high PKA activity.

We then addressed the question whether Cdc20 function may be affected by PKA activity. We argued that if this were the case, then the inhibitory effect of activated $Ras2^{Val19}$ on *apc* mutants may be reduced by the overexpression of *CDC20*. To test this, a *cdc27-1* mutant containing both $RAS2^{Val19}$ and *GAL–CDC20* on centromeric plasmids was pregrown in raffinose medium at 25 °C, streaked onto either raffinose or galactose plates and incubated at 30 °C. Microscopic examination of cells showed that high levels of Cdc20 allowed many cells to form colonies (Fig. 6d), albeit distinctly more slowly than wild-type cells. Thus, high levels of Cdc20 partially suppress the inhibitory influence of activated Ras signalling on the viability of *cdc27-1* mutants. These results provide evidence that the activation of the cAMP/PKA pathway affects APC/C, at least in part, via the Cdc20 regulatory protein.

DISCUSSION

The activity of the anaphase-promoting complex/cyclosome (APC/C) is controlled during the cell cycle by a variety of positive and negative regulators (Harper *et al.*, 2002; Peters, 2002; Zachariae & Nasmyth, 1999). One of these regulatory factors is cAMP-dependent protein kinase, also known as protein kinase A (PKA), which inhibits APC/C activity in yeast and mammalian cells.

In *S. cerevisiae*, PKA can be stimulated by a shift to glucose medium or by the activation of Ras proteins (Thevelein & de Winde, 1999). We have presented genetic data showing that glucose and dominantly active $Ras2^{Val19}$ proteins, the equivalent to the oncogenic mammalian Ras^{Val12}, severely reduce the viability of *apc* mutants in a synergistic manner. We suggest that the expression of the $RAS2^{Val19}$ allele in combination with growth on glucose medium causes an efficient activation of adenylate cyclase, resulting in enhanced PKA activity and potent inhibition of APC/C. Our findings are consistent with the model proposing that glucose stimulates adenylate cyclase independently of Ras1 and Ras2 (Colombo *et al.*, 1998).

We also showed that APC/C inhibition in response to activated Ras2 proteins seems to occur exclusively via cAMP and PKA, but not by the MAPK pathway. Furthermore, we found that Tpk1, Tpk2 and Tpk3 have overlapping roles in APC/C inhibition, suggesting that each of the Tpk proteins is capable of taking over this function in response to a shift to glucose medium. However, our data provide evidence that Tpk2 performs this function more efficiently than Tpk1 or Tpk3 (Fig. 4d). This effect might be explained by the findings that these kinases have distinctly different catalytic activities (Mazon *et al.*, 1993; Toda *et al.*, 1987; Zähringer *et al.*, 1998). Indeed, Tpk2 was shown to have higher catalytic activity than Tpk1 and Tpk3. For Tpk3, this is apparently due to its poor expression (Mazon *et al.*, 1993). This is consistent with our data, showing that *TPK3*, similar to *TPK2*, mediates efficient APC/C inhibition when overexpressed (Fig. 5). In conclusion, Tpk proteins have overlapping functions in APC/C inhibition but obviously have different efficiences at normal expression levels.

Genetic and biochemical data suggest that PKA-mediated inhibition of APC/C is conserved in eukaryotes from yeast to mammals (Anghileri *et al.*, 1999; Kotani *et al.*, 1998, 1999; Yamada *et al.*, 1997; Yamashita *et al.*, 1996). In budding and fission yeast, it remains to be shown whether PKA directly phosphorylates APC/C subunits, as shown *in vitro* with the reconstituted mammalian APC/C (Kotani *et al.*, 1998). Most yeast APC/C subunits contain consensus phosphorylation sites for PKA (Kennelly & Krebs, 1991). Remarkably, 28 potential sites were found in the Apc1 subunit, and 7–8 sites in three other subunits. An important task in the future will be to find out how PKA inhibits APC/C activity. In mammalian cells, it was shown that the activator protein Cdc20 was unable to bind APC/C when the complex was preincubated with PKA. Thus, PKA may inhibit APC/C function by modifying critical subunits required for the binding of Cdc20, thereby preventing its association with the core complex. Our results showing that high levels of Cdc20 partially suppress the inhibitory effect of $RAS2^{Val19}$ (Fig. 6d) are consistent with such a model.

It will be an interesting task to elucidate which intra- or extracellular signals regulate PKA during mitosis. The growth medium appears to be one of these signals (Anghileri *et al.*, 1999; Irniger *et al.*, 2000). The availability of rich carbon sources such as glucose may cause a delay in the progression through mitosis, by activation of PKA and inhibition of APC/C. Such a model is consistent with the findings that daughter cells are born at larger cell size on rich medium (Alberghina *et al.*, 1998). PKA-mediated inhibition of APC/C may also be a mechanism for the delay in mitosis

during pseudohyphal growth (Kron *et al.*, 1994; Rua *et al.*, 2001). Other intra- or extracellular signals may be transmitted by Ras proteins. Taken together, the cAMP/PKA pathway represents a suitable system for the integration of multiple signals which are then communicated to the cell cycle machinery.

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