Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast

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Cyclin-dependent kinase inhibitors (CKIs) play key roles in controlling the eukaryotic cell cycle by coordinating cell proliferation and differentiation. Understanding the roles of CKIs requires knowledge of how they are regulated both through the cell cycle and in response to extracellular signals. Here we show that the yeast CKI, Far1p, is controlled by ubiquitin-dependent proteolysis. Wild-type Far1p was stable only in the G1 phase of the cell cycle. Biochemical and genetic evidence indicate that its degradation required the components of the G1-S ubiquitination system, Cdc34p, Cdc4p, Cdc53p, and Skp1p. We isolated a mutant form of Far1p (Far1p-22) that was able to induce cell cycle arrest in the absence of α-factor. Cells that overexpress Far1-22p arrested in G1 as large unbudded cells with low Cdc28p–Cln3p kinase activity. Wild-type Far1p, but not Far1-22p, was readily ubiquitinated in vitro in a CDC34- and CDC4-dependent manner. Far1-22p harbors a single amino acid change, from serine to proline at residue 87, which alters phosphorylation by Cdc28p–Cln2p in vitro. Our results show that Far1p is regulated by ubiquitin-mediated proteolysis and suggest that phosphorylation of Far1p by the Cdc28p–Cln3p kinase is part of the recognition signal for ubiquitination.

Key Words: Ubiquitin; CKI; degradation; cell cycle; pheromone response; Far1p

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The eukaryotic cell cycle is driven by the sequential activation and inactivation of cyclin-dependent kinases (Cdks) (Nigg 1995). Cdks are composed of a catalytic subunit (Cdc28p in budding yeast) and a cyclin regulatory subunit (Nasmyth 1993). The protein kinase activity of the complex is further regulated by specific phosphorylation of the catalytic subunit and by association with Cdk inhibitors (Morgan 1995). CKIs have been identified in a variety of organisms, playing key roles during development, cell cycle regulation, and as effectors of checkpoint mechanisms (Sherr and Roberts 1995; Harper and Elledge 1996). Some of these inhibitory proteins are also implicated in the development of cancer (Hunter and Pines 1994; Sherr 1996). Two CKIs have been identified in budding yeast Saccharomyces cerevisiae: p40Sic1 regulates entry into S phase by inhibiting the Cdc28p–Clb5p kinases (Mendenhall 1993; Schwob et al. 1994) and Far1p is specifically required to arrest the cell cycle in response to pheromones (Peter and Herskowitz 1994b). Mating pheromones activate the mitogen-activated protein (MAP) kinase signal transduction pathway that induces changes in gene transcription, alterations of cellular morphology, and cell cycle arrest in G1 (Sprague and Thorner 1992; Herskowitz 1995; Leberer et al. 1997). Cell cycle arrest is mediated by Far1p, which functions as a CKI to inhibit the Cdc28p–Cln3p kinase (Peter and Herskowitz 1994b).

Despite the importance of CKIs, little is known about their regulation (Peter 1997). In the case of Far1p, the mRNA levels fluctuate throughout the cell cycle, peaking in G1 (Mckinney et al. 1993). Far1p levels increase several-fold in response to mating pheromones, and the protein becomes rapidly phosphorylated by the MAP kinase Fus3p (Peter et al. 1993). Although transcriptional induction of FAR1 in response to pheromones is necessary to cause cell cycle arrest, it is not sufficient (Chang and Herskowitz 1992; Peter and Herskowitz 1994a), indicating that post-translational modification is also required to activate Far1p. The CKI p40Sic1 appears to be regulated through the cell cycle by ubiquitin-dependent degradation (Schwob et al. 1994a). Likewise, the mammalian inhibitor p27Kip1 has been shown to be ubiquitinated in vitro and in vivo (Pagano et al. 1995).

Ubiquitin-dependent degradation of proteins has emerged as a key mechanism for regulating cell cycle transitions. Destruction is triggered by the covalent at-
attachment of ubiquitin, which targets proteins for degradation by the proteasome (Ciechanover 1994; Hochstrasser 1996). At least two distinct ubiquitination complexes regulate cell cycle-dependent destruction of target proteins. Cdc16p, Cdc23p, and Cdc27p are part of a large multisubunit complex, termed anaphase promoting complex (APC), which is required for the onset of anaphase by degrading mitotic cyclins and as yet unknown proteins (King et al. 1996). Cdc34p, Cdc4p, Cdc53p, and Skp1p are required for cells to enter S phase, and cells lacking the function of any of these proteins arrest with a G1 DNA content and an elongated bud (Nasmyth 1996). CDC34 encodes a ubiquitin-conjugating enzyme (Goebel et al. 1988), whereas little information is available on the molecular function of the other proteins. Cdc4p contains two sequence motifs found in many unrelated proteins: an F-box, which mediates the interaction with Skp1p, and several WD40 repeats that may be related proteins: an F-box, which mediates the interaction with Skp1p, and several WD40 repeats that may be related to other proteins (Goebl et al. 1988). Although it is not yet understood how p40Sic1 is specifically recognized by the ubiquitination machinery, recent experiments suggest a role for phosphorylation by the Cdc28p-Clnp kinase (Schneider et al. 1996; Tyers 1996).

To study the post-translational regulation of the CKI Far1p, we have isolated a mutant Far1p (Far1-22p), which arrests cells even in the absence of mating pheromones (Peter and Herskowitz 1994a). Whereas wild-type Far1p was only present in the G1-phase, Far1-22p was found in the nucleus throughout the cell cycle. Our results strongly suggest that Far1-22p arrests cells in G1 because it can no longer be ubiquitinated and degraded by the G1-S degradation system. Sequencing of Far1-22p revealed that the stabilizing mutation changed Serine 87 (Ser-87) to a proline residue. Interestingly, Ser-87 was located in a consensus phosphorylation site for Cdc28p kinase and could be specifically phosphorylated by Cdc28p-Cln2p in vitro. Our results show that Far1p is regulated by ubiquitin-dependent proteolysis, and suggest that an important determinant of the recognition signal for ubiquitination is a specific phosphorylation of Far1p by the Cdc28p-Clnp kinase.

Results

Cells producing Far1-22p arrest their cell cycle by inhibiting the Cdc28p-Clnp kinase in the absence of the mating pheromone pathway

Transcription of the CKI Far1p is induced several-fold in response to mating pheromones by a process that requires the transcriptional activator Ste12p (Chang and Herskowitz 1990; Oehlen et al. 1996). Transcriptional induction of Far1p, however, is not sufficient to cause cell cycle Arrest: cells that produce wild-type Far1p from the inducible GAL1 promoter grow normally (Fig. 1; Chang and Herskowitz 1992). In an attempt to investigate the mechanism of the post-transcriptional activation of Far1p, we isolated an allele of FAR1 (Far1-22) that was able to inhibit cell division in the absence of α-factor (Fig. 1A). Cells expressing Far1-22p from the GAL1 promoter were unable to form colonies in the presence of galactose (which activates the GAL1 promoter), but grew normally on medium containing glucose (Fig. 1A,B; data not shown). To determine whether the function of Far1-22p requires basal activity of the mating pathway, growth inhibition was examined in aα diploid cells that are unable to respond to pheromones (Johnson 1995): Like haploid cells, aα diploids were inhibited by Far1-22p (Fig. 1A) showing that Far1-22p was...
able to prevent cell proliferation, not only in the absence of α-factor, but also in cells lacking a functional pheromone response. Consistent with these results, Far1-22p inhibited proliferation of cells deleted for various components of the mating pathway such as FUS3 (Fig. 1B), STE11, or STE7 (data not shown).

Next, we examined the phenotype of cells arrested by Far1-22p. After synthesis of Far1-22p was induced for 6 hr, ~90% of the cells accumulated as large unbudded cells, indicating that they arrested in the G1 phase of the cell cycle before bud emergence (Fig. 1C, top left). These cells contained predominantly one nucleus as visualized by 4′, 6-diamidino-2-phenylindole (DAPI) (data not shown) and a 1N DNA content as determined by fluorescence-activated cell sorting (FACS) analysis (Fig. 1D). Staining the cells with rhodamine phalloidin revealed that the actin cytoskeleton was unpolarized, indicative of cells arrested at Start (Fig. 1C, bottom left panel). In contrast, cells producing wild-type Far1p continued to bud normally (Fig. 1C, top right panel) and displayed polarized actin (Fig. 1C, bottom right panel; Lew and Reed 1995). In addition, cells arrested by overexpression of Far1-22p were able to fully induce a FUS1–lacZ reporter construct in response to α-factor (data not shown), suggesting that they were blocked in a mating-competent state (Oehlen and Cross 1994). Taken together, these observations suggest that cells producing Far1-22p arrest at Start in the G1 phase of the cell cycle.

The transition from G1 to S phase requires activation of the G1 cyclin-dependent kinase Cdc28p–Cln1p (Nasmyth 1993). Whereas the Cdc28p catalytic subunit is present at constant levels throughout the cell cycle, the levels of the G1 cyclins Cln1p and Cln2p peak as cells pass through Start (Koch and Nasmyth 1994). Cells arrested by overexpression of Far1-22p contained high levels of Cln2p (Fig. 2B), showing that Far1-22p did not prevent synthesis of Cln2p. In contrast, cells arrested by α-factor repress transcription of CLN1 and CLN2 (Wittenberg et al. 1990). Thus, our results indicate that repression of CLN1 and CLN2 transcription is not directly caused by Far1p, but rather by an α-factor-induced event. In response to α-factor, Far1p has been shown previously to bind directly and thereby inhibit the activity of Cdc28p associated with the G1 cyclins Cln1p and Cln2p (Peter and Herskowitz 1994b). As shown in Figure 2A, the histone H1 kinase activity associated with Cln2p

Figure 2. Cells producing Far1-22p arrest in G1 by inhibition of the G1 kinase, Cdc28p–Cln1p. (A,B) Cln2p tagged at its carboxyl terminus with three copies of the HA epitope (Cln2-HA) was immunoprecipitated from extracts prepared from cells (YMT263) expressing either Far1-22p or for control, an inactive mutant form of Far1p, Far1-22/Δ285–350, from the inducible GAL promoter. Cells were grown in media containing galactose (GAL, GAL promoter on) or glucose (GLU, GAL promoter off). Cln2-HA-associated kinase activity was measured with histone H1 as a substrate and quantified (A). The kinase activity associated with Cln2-HA from cells grown in glucose was normalized to 100%. Similar amounts of Cln2-HA were immunoprecipitated in each assay as shown by immunoblotting (B). (C) Growth inhibition caused by production of Far1-22p was dependent on the ability of Far1p to bind to the Cdc28p–Cln1p kinase. The following Far1p proteins were analyzed: wild-type Far1p, Far1-22p, Far1-Δ285–350, which is unable to bind to Cdc28p–Cln1p (Peter et al. 1993), and Far1-22/Δ285–350. (D) Growth inhibition caused by expression of Far1-22p was rescued by co-overexpression of a stable G1-cyclin, Cln3p (DAF1). DAF1-1 cells (IH2517), which express a stable form of the G1 cyclin Cln3p or isogenic wild-type cells (IH2518), were transformed with plasmids expressing either wild-type Far1p, Far1-22p, or Far1-22/Δ285–350p from the inducible GAL promoter.
was reduced in cells arrested with Far1-22p when compared with cells expressing either wild-type Far1p (data not shown) or an inactive mutant of Far1-22p (Far1-22/Δp). The Cln2p-associated kinase activity was not reduced when synthesis of Far1-22p was not induced (Fig. 2A). Binding of Far1p and Cdc28p requires a segment between amino acids 285 and 350 of Far1p (Peter et al. 1993). Cells producing Far1-22p deleted for this segment (Far1-22/Δp) failed to inhibit cell division (Fig. 2C), suggesting that binding of Far1-22p and Cdc28p kinase was required to arrest the cell cycle. Finally, the effect of Far1-22p could be suppressed by high levels of G1-cyclins: Cells overexpressing a truncated form of Cln3p (Cross 1988) were able to grow in the presence of Far1-22p (Fig. 2D). Taken together, these results suggest that Far1-22p, at least in part, arrests cells at Start by inhibiting the Cdc28p–Clnp kinase in the absence of α-factor.

Far1-22p has an increased half-life and is stable throughout the cell cycle

The presence of wild-type Far1p is restricted to the G1 phase of the cell cycle. This pattern is ensured by regulated transcription of FAR1 and by proteolysis (McKinney and Cross 1995). Because we found that steady-state levels of Far1-22p were higher than wild-type Far1p (data not shown), we compared the stability of wild-type Far1p with Far1-22p. Both proteins were expressed from the GAL1 promoter, at which time synthesis was turned off by addition of glucose. We observed rapid degradation of wild-type Far1p with a half-life of ∼30 min (Fig. 3A,B). In contrast, Far1-22p remained stable throughout the time course (half-life ∼120 min), showing that Far1-22p harbors a specific alteration that stabilizes the protein in vivo.

Next, we examined whether Far1p was degraded in a cell cycle-dependent manner. To drive FAR1 transcription through the cell cycle, we expressed Far1p from the inducible GAL1 promoter. Cells were arrested in S phase with hydroxyurea (HU) or in mitosis with the microtubule depolymerizing drug nocodazole (Noc). Synthesis of either wild-type Far1p or Far1-22p was then induced by addition of galactose and analyzed by immunoblotting. Little wild-type Far1p could be detected in cells arrested in S phase or in mitosis (Fig. 3C). The remaining low levels of Far1p were most likely the result of incomplete cell cycle arrest (90% arrest in both cases). In contrast, Far1-22p was present at high levels in both S and M phases of the cell cycle (Fig. 3C). The finding that Far1-22p was present in a cell cycle-independent manner also showed that FAR1 RNA was present throughout the cell cycle when driven from the GAL1 promoter. Thus, we conclude that wild-type Far1p, but not Far1-22p, was rapidly degraded at cell cycle stages other than G1. The degradation system for Far1p appears, therefore, to be active once cells pass through Start and remains active throughout the cell cycle until the cells exit from mitosis.

**Figure 3.** Far1-22p has an increased half-life and is present throughout the cell cycle. (A) Cells (K699) that carry a plasmid coding for either wild-type Far1p–GFP or Far1-22p–GFP from the inducible GAL1 promoter were grown in raffinose and expression was induced by addition of galactose. Control cells were treated identically except that they were not exposed to the drugs (expo). After 5 hr, the cell cycle was arrested in S phase with hydroxyurea (HU) or in mitosis with nocodazole (Noc), and then expression was induced by addition of galactose. Control cells were treated identically except that they were not exposed to the drugs (expo). After 5 hr, cells were analyzed for the presence of Far1p–GFP by immunoblotting with antibodies specific for GFP (top panel). Equal loading was confirmed by immunoblotting the samples with antibodies against actin (bottom panel).
We have also studied the presence of Far1p during the cell cycle by use of fusions of either wild-type Far1p or Far1-22p to the green fluorescent protein (GFP). The Far1p-GFP fusion protein was functional and able to restore both cell cycle arrest and mating competence to cells lacking Far1p (data not shown). As shown in Figure 4, wild-type Far1-GFP protein was readily visualized in the nucleus of un budded G1 cells, but very little Far1p was detected in cells that had passed Start (Fig. 4, top row). These results confirmed that wild-type Far1p was degraded after Start even when continuously expressed from the GAL1 promoter. Prolonged induction of Far1-22p-GFP resulted in accumulation of the cells at Start. Far1-22p-GFP was visualized in the nucleus, showing that the stabilizing mutation that affects Far1-22p did not interfere with the subcellular localization of the protein. Interestingly, however, Far1-22p-GFP was present in the nucleus of cells at all stages of the cell cycle (Fig. 4, bottom row), confirming that Far1-22p was not degraded after cells passed Start.

Degradation of Far1p is required to efficiently re-enter the cell cycle after α-factor arrest

To address the physiological significance of Far1p degradation, we asked whether degradation of Far1p was required for cells to re-enter the cell cycle after arrest by α-factor. Cells deleted for FAR1, but producing Far1-22p from its own promoter, were able to grow normally because α-factor is required for full transcriptional induction of FAR1 (Chang and Herskowitz 1990; Oehlen et al. 1996). These cells were hypersensitive to α-factor, forming a halo that was larger than that of cells expressing wild-type Far1p (Fig. 5A, top panels), showing that Far1-22p was functional. Interestingly, halos formed by cells expressing Far1-22p failed to fill in (Fig. 5A, bottom panel), indicating that these cells were unable to efficiently resume cell division. To further examine recovery from α-factor arrest, far1Δ cells producing either wild-type Far1p or Far1-22p were monitored microscopically as they synchronously re-entered the cell cycle. As shown in Figure 5 (B, open circles), cells producing wild-type Far1p started to bud after an initial lag phase, indicating that they resumed cell division and entered S phase. In contrast, cells producing nondegradable Far1-22p failed to re-enter the cell cycle and remained unbudded (Fig. 5B, filled squares). Determination of Far1p levels by immunoblotting revealed that wild-type Far1p rapidly disappeared at the time of bud emergence (Fig. 5B), whereas Far1-22p remained constant and accumulated in slower migrating forms (Fig. 5C). This shift in mobility of Far1p was a consequence of phosphorylation, because incubation with alkaline phosphatase (CIP) eliminated the slower migrating forms (data not shown). Taken together, these results thus confirmed that Far1p was degraded when cells pass through Start and further indicated that degradation of Far1p is required for cells to efficiently re-enter the cell cycle after α-factor arrest.

Far1p is stabilized in cells harboring temperature-sensitive alleles in components of the G1–S ubiquitination system

Next, we examined whether Far1p degradation requires the G1–S degradation system. Because overexpression of Far1-22p, which cannot be degraded, causes cell cycle arrest, we reasoned that overexpression of wild-type Far1p in a cell that exhibited a reduced ability to degrade Far1p might similarly cause cell cycle arrest in G1. Consistent with this hypothesis, we found that overexpression of Far1p in a cdc34 mutant caused inviability at the semipermissive temperature (Fig. 6A). This effect was specific as wild-type cells or cells harboring a temperature-sensitive allele of CDC16, which is required to degrade mitotic cyclins (Irniger et al. 1995), grew normally when overexpressing Far1p. Examination of cdc34 cells overexpressing Far1p revealed that they arrested as large, unbudded cells (~90% after 6 hr of induction of Far1p by addition of galactose). In contrast, cdc34 mutant cells harboring control constructs accumulated prior to DNA replication with large elongated buds (Fig. 6B, top row). Staining of the actin cytoskeleton with rhodamine phal-
loidin showed that cdc34 cells producing Far1p were unpolarized, indicating that they were arrested at Start (Fig. 6B, bottom row). Thus, the morphology of these cells appeared indistinguishable from wild-type cells arrested by stabilized Far1-22p (Fig. 1C), suggesting that Far1p is degraded by a Cdc34p-dependent mechanism.

Figure 5. Degradation of Far1p is required for cells to recover efficiently from α-factor induced cell cycle arrest. (A) Cell cycle arrest was assayed by halo formation of far1Δ cells transformed with single-copy plasmids carrying wild-type FAR1, FAR1-22, FAR1-60F3 (Peter et al. 1993), or no insert. (Top two rows) bar1Δ cells (K2180), (bottom row) BAR1+ cells (YMP1054). Filter disks contain 0.1 µg (top two rows) and 10 µg of α-factor (bottom row). Note that cells producing Far1-22p are hypersensitive to pheromone and that halos fill in inefficiently. (B,C) Cells producing Far1-22p are unable to re-enter the cell cycle. Cells deleted for FAR1 (K2180) producing wild-type Far1p or Far1-22p from the GAL promoter were grown in raffinose medium to an OD600 of 0.4, at which time cells were arrested by the simultaneous addition of α-factor and galactose. After 3 hr, cells were washed and reinoculated in fresh medium, without α-factor, but containing galactose. Every 30 min, samples were harvested. Re-entry into the cell cycle was monitored microscopically by analyzing the percentage of unbudded cells (B) Levels of wild-type Far1p (bottom panel) and Far1-22p (top panel) were determined after immunoblotting extracts with Far1p antibodies (C, lanes 1–7). Note that in contrast to cells producing wild-type Far1p, Far1-22p is accumulating in G1 and cells are unable to form a bud.

Figure 6. Overexpression of wild-type Far1p is lethal in cdc34 cells at the semipermissive temperature because of stabilization of the Far1 protein. (A) Temperature-sensitive cdc16 (K4102) and cdc34 (YMT670) mutants, as well as isogenic wild-type cells (K699), were transformed with a plasmid carrying wild-type FAR1 under the control of the inducible GAL promoter or an empty vector (vec) for control. Transformants were plated on media containing galactose and incubated at 30°C. Wild-type and cdc16 mutant strains tolerate overexpression of Far1p, whereas cdc34 mutants do not. All strains grow normally when plated on medium containing glucose (data not shown). (B) cdc34 cells (YMT670) carrying a plasmid expressing Far1p from the inducible GAL promoter (pFar1; left panels) or control plasmid with no insert (vec; right panels) were grown in raffinose at 30°C, at which time expression of Far1p was induced by addition of galactose. After 5 hr, cells were fixed and analyzed by phase contrast microscopy (Phase, top panels) or after actin staining with rhodamine phalloidin (Actin, bottom panels). cdc34 cells producing Far1p arrest with a morphology indistinguishable from that of wild-type cells producing Far1-22p (Fig. 1C). (C) The half-life of wild-type Far1p was determined in cdc34 far1Δ (top panel; YMP1056) and far1Δ cells (bottom panel; YMP1054), which express Far1p from the GAL promoter. After repression of the GAL promoter at the indicated times (minutes), aliquots were removed and examined by immunoblotting as described. The specificity of the Far1p antibodies was confirmed by analysis of cells carrying an empty vector (vec; lane 1). The position of Far1p is marked by an arrowhead; the asterisks mark an unspecific protein recognized by the Far1p antiserum.
In addition to Cdc34p, the CDC53, CDC4, and SKP1 gene products have also been implicated in the ubiquitin-dependent degradation of target proteins (Nasmyth 1996; Deshaies 1997). Overexpression of Far1p was lethal in any of these backgrounds at the semi-permissive temperature, suggesting that these components were required for degradation of Far1p (Fig. 7A; data not shown). Specific temperature-sensitive alleles of SKP1 that arrest cells either before DNA replication in G1 or before mitosis in G2 (Bai et al. 1996; Connelly and Hieter 1996) have been identified. Interestingly, overexpression of Far1p was able to arrest cells harboring the G1-specific allele of SKP1 but had no effect on cells harboring the G2-specific allele of SKP1 (Fig. 7A). Taken together, these results indicate that Far1p is degraded by the G1–S ubiquitination system involving the ubiquitin-conjugating enzyme Cdc34p and the proteins Cdc53p, Cdc4p, and Skp1p.

Consistent with the proposed role of Cdc34p in the degradation of Far1p, the half-life of wild-type Far1p was dramatically increased in cdc34 and cdc53 cells at the nonpermissive temperature (Fig. 6C; data not shown). To determine whether the cell cycle stage, rather than lack of Cdc34p function, accounted for the accumulation of Far1p, we analyzed Far1p levels in cdc34 cells that were simultaneously deleted for SIC1. These cells are able to replicate normally but arrest subsequently in G2, presumably because they fail to degrade an as yet unknown target (Schwob et al. 1994). We expressed Far1p fused at its carboxyl terminus to LacZ from the inducible GAL promoter, which facilitated quantification of Far1p levels by a color assay (Barral et al. 1995). The Far1–LacZ fusion protein, like wild-type Far1p, was degraded in a cell cycle-dependent manner (Fig. 7B,C; B. Catarin and M. Peter, unpubl.). As expected, Far1p accumulated in cdc34 cells, and was present at levels approximately three- to fourfold higher than in isogenic wild-type cells or cells lacking Sic1 (Fig. 7B; McKinney et al. 1993). Importantly, Far1p levels remained high in cdc34 sic1 cells, whereas low levels of Far1p were detected in cdc16 cells, which arrest in mitosis. In contrast, the Far1–22–LacZ fusion protein was present at higher levels than wild-type Far1–LacZ protein in both wild-type and cdc16 cells (Fig. 7C) whereas the levels of Far1–LacZp and Far1–22–LacZp were comparable when produced in cdc34 or cdc34 sic1 cells (Fig. 7C). Taken together, these results show that lack of Cdc34p itself, rather than arrest at a particular stage of the cell cycle, was responsible for the accumulation of Far1p.

In vitro ubiquitination of Far1p depends upon Cdc34p, Cdc4p, and cyclins

To determine whether Far1p is ubiquitinated by the G1–S ubiquitination system, we in vitro-translated Far1p in rabbit reticulocyte lysates (Fig. 8A) or wheat germ extracts (Fig. 8B) in the presence of [35S]methionine and added DEAE-fractionated yeast extracts (Verma et al. 1997a). These extracts are devoid of the G1 cyclins Cln1p, Cln2p, and Cln3p and are depleted of Cdc34p (Fig. 8A) or lack functional Cdc4p (Fig. 8B). Addition of Far1p, purified GST–Cln2p, ubiquitin (Ub), and Cdc34p resulted in accumulation of slower migrating forms of Far1p (Fig. 8A, lane 2). Methylated ubiquitin (mEuB), which blocks formation of mult ubiquitin chains by preventing Ub–Ub ligation (Hershko and Heller 1985), pre-

Figure 7. Overexpression of Far1p is lethal in skp1 mutants in an allele-specific fashion and Far1p accumulates in cdc34 mutants independent of the cell cycle stage. (A) The effect of overexpression of Far1p was tested in specific temperature-sensitive alleles of SKP1 that either cause arrest in G1 (skp1-11, Y552) or in G2 (skp1-12, Y554) as described in Fig. 6. Only cells harboring the G1-specific allele of SKP1 were arrested by overexpression of wild-type Far1p. (B) Far1p accumulates in cdc34 (YMT670) and cdc34 Δsic1 cells (ES464). The indicated strains were arrested at 37°C and the accumulation of a Far1–LacZ fusion protein was quantified and plotted as Miller Units deduced from three independent experiments. Note that Far1–LacZp accumulates in cdc34 mutants independently of the cell cycle arrest point; no accumulation was observed in cdc16 cells or cells deleted for Sic1. (C) Far1–22–LacZp accumulates in wild-type (K699) and cdc16 (K4102) mutant cells but is produced at levels similar to wild-type Far1–LacZp in cdc34 (YMT670) and cdc34 Δsic1 (ES464) cells.
Ubiquitination of wild-type Far1p was dependent on Cdc4p (lanes 6–10). No ubiquitination was observed with Far1-22p (lanes 1–5). Reduced accumulation of high molecular weight ubiquitin conjugates was observed if methyl-ubiquitin was added to block chain extension (lane 3). No ubiquitination was observed with Far1-22p (lanes 6–10). Ubiquitination of wild-type Far1p was dependent on Cdc4p (lanes 1 and 4). Ubiquitination in cdc4 extracts was restored only by the simultaneous addition of purified Cdc34p and baculovirus-infected insect cell lysate containing Cdc4p (lane 3). The addition of insect lysate containing Cdc28p serves as a specificity control (lane 4).

Figure 8. Wild-type Far1p but not Far1-22p is ubiquitinated in vitro. Wild-type Far1p (A, lanes 1–5; B, lanes 1–4) or Far1-22p (A, lanes 6–10) synthesized in vitro in the presence of [35S]methionine was incubated in fractionated extracts prepared from cells lacking CLN1,2, and 3 (A) or cells deleted for CLN1,2, and 3, which are also temperature-sensitive for cdc4 (B). These extracts are devoid of Cdc34p and contain low levels of ubiquitin (Verma et al. 1997a). (A) Wild-type Far1p was ubiquitinated if the extract was supplemented with the addition of Cdc34p, GST–Cln2p, and ubiquitin (lane 2). Reduced accumulation of high molecular weight ubiquitin conjugates was observed if methyl-ubiquitin was added to block chain extension (lane 3). No ubiquitination was observed with Far1-22p (lanes 6–10). (B) Ubiquitination of wild-type Far1p was dependent on Cdc4p (lanes 1 and 4). Ubiquitination in cdc4 extracts was restored only by the simultaneous addition of purified Cdc34p and baculovirus-infected insect cell lysate containing Cdc4p (lane 3). The addition of insect lysate containing Cdc28p serves as a specificity control (lane 4).

Discussion

Far1p is degraded in a cell cycle-dependent manner

Far1p accumulates during the G1 phase of the cell cycle. This expression pattern is achieved in part by transcriptional regulation of FAR1, which is restricted to G1 and late M phase of the cell cycle (McKinney et al. 1993). We have found that even when Far1p is expressed from constitutive promoters, however, it is still detectable only in G1 cells, because Far1p is rapidly degraded at stages other than G1. The observation that a nondegradable mutant form of Far1p is present throughout the cell cycle excludes the possibility that translation of FAR1 RNA is restricted to G1, as is the case for the mammalian CKI p27 (Hengst and Reed 1996). We show that Far1p is de-
Far1p is a yeast CKI that is required to arrest the cell cycle in the presence of mating pheromones but is not necessary for normal cell cycle progression (Chang and Herskowitz 1990). The degradation of Far1p is physiologically relevant during yeast mating. Cells arrested with \( \alpha \)-factor that are unable to find a mating partner adapt to the presence of mating pheromones and resume cell division (Sprague and Thorner 1992). Re-entry into the cell cycle requires the degradation of Far1p: Cells that produce nondegradable Far1p were unable to properly activate the Cdc28p–Clnp kinase after release from \( \alpha \)-factor and, as a consequence, failed to recover efficiently.

Far1p degradation by the G1–S ubiquitination system requires phosphorylation of Ser-87

Two ubiquitin-dependent degradation systems trigger key transitions during the eukaryotic cell cycle. The onset of S phase is initiated by the proteolysis of p40\(^{G_{1}}\)cicl, which involves the G1–S ubiquitination system, whereas the onset of anaphase requires the anaphase-promoting complex (APC), which degrades inhibitors of sister chromatid separation (Nasmyth 1996). Substrates for the APC typically contain a short sequence termed the destruction box, which is both necessary and sufficient for degradation (King et al. 1996). Substrates for the G1–S ubiquitination system do not contain such a destruction box, and it is currently unknown how these proteins are recognized. The amino-terminal 50 amino acid residues of Far1p are required for its degradation in vivo (Mckinney and Cross 1995). It is unlikely, however, that these 50 amino acid residues comprise a destruction signal per se, but they contain a functional nuclear localization signal and a truncated Far1p protein lacking these 50 amino acids found predominantly in the cytoplasm (M. Peter, unpubl.). Because Cdc34p is localized in the nucleus (Goebel et al., 1994), cytoplasmic localization may result in stabilization of Far1p. In contrast, failure to degrade Far1-22p is not caused by mislocalization, because Far1-22p still localized to the nucleus. Recent evidence suggests that phosphorylation might provide a signal for degradation at the G1 to S transition and remains unstable until cells exit from mitosis. Cells overexpressing a form of Far1p that fails to be degraded accumulate predominantly at Start and not in S phase or mitosis, which is consistent with previous in vitro assays indicating that Far1p inhibits Cdc28p complexed with G1 cyclins but is a poor inhibitor of S phase or M phase Cdks (Peter and Herskowitz 1994b).
ubiquitination and degradation by the G₁-S destruction system (Deshayes 1997). Mutations that inactivate phosphorylation sites of the G₁ cyclin Cln2p in yeast or mammalian cyclin E stabilize these proteins in vivo (Clurmann et al. 1996; Lanker et al. 1996; Won and Reed 1996). Furthermore, phosphorylated, but not unphosphorylated, Cln2p specifically interacts with Cdc53p (Willems et al. 1996). Several lines of evidence indicate that degradation of Far1p also requires phosphorylation that may be achieved by the Cdc28p–Cln1p kinase. First, degradation of Far1p occurs when cells progress through Start, concomitant with the activation of the Cdc28p–Cln1p kinase. Second, Far1p is phosphorylated in vitro and in vivo by Cdc28p–Cln1p kinase (Peter et al. 1993; Tyers and Futcher 1993). Third, Far1p is stable in cells harboring a temperature-sensitive mutation of CDC28 (Oehlen and Cross 1994; M. Peter and S. Henchoz, unpublished). Fourth, we have shown that ubiquitination of Far1p in vitro requires active Cdc28p–Cln1p kinase. Finally, mutation of a site phosphorylated by Cdc28p–Cln1p kinase stabilizes Far1p in vivo and prevents its ubiquitination. Taken together, we propose the following model for the degradation of Far1p (Fig. 10). Far1p binds to the Cdc28p–Cln1p kinase, which in turn phosphorylates Far1p on Ser-87 and possibly other residues. Phosphorylated Far1p is then recognized by the G₁-S ubiquitination system composed of Cdc34p, Skp1p, Cdc53p, and Cdc4p, which ubiquitinates Far1p. Ubiquitinylated Far1p is subsequently degraded by the 26S proteasome. It remains to be shown whether Far1p is ubiquitinated in vivo. Despite the use of a mutant ubiquitin (UbK48R, G76A), which cannot be cleaved by ubiquitin-deconjugating enzymes (Hodgins et al. 1992; Willems et al. 1996), we were unable, so far, to convincingly show covalently attached ubiquitin on Far1p in vivo. We cannot, therefore, rigorously exclude the possibility that degradation of Far1p in vivo does not require ubiquitination of Far1p itself but might involve a ubiquitinylated protein that targets Far1p to the proteasome.

A similar pathway has been proposed for controlling degradation of the G₁ p40Sic1 (Deshayes 1997). Analogous to Far1p, an essential requirement for ubiquitination and degradation of p40Sic1 appears to be phosphorylation by the Cdc28p–Cln1p kinase, thereby ensuring that Sic1 cannot be initiated before the G₁-specific kinase Cdc28p–Cln1p (or Cln2p) has been activated (Tyers 1996; Schneider et al. 1996; Verma et al. 1997b). Whereas available results strongly support a function of the Cdc28p–Cln1p kinase in the recognition of the substrate, they do not exclude the possibility that Cdc28p–Cln1p kinase might also directly regulate the activity of the G₁-S ubiquitination system. At least two lines of evidence, however, argue against the latter interpretation. First, it is unlikely that Cdc28p–Cln1p is required to activate a component in the extract other than the substrate because p40Sic1 is ubiquitinylated in vitro in the absence of Cln2p provided that p40Sic1 is phosphorylated by Cdc28p–Cln1p prior to addition to the extract (R. Verma and R.J. Deshares, unpublished). Second, mutations in Far1p that prevent phosphorylation by Cdc28p–Cln1p kinase also disrupt ubiquitination, suggesting that phosphorylation of the substrate is essential for ubiquitination.

The activity of the Cdc28p–Cln1(2)p kinase is restricted to an interval between Start and the initiation of S phase, and thus correlates with the abrupt disappearance of Far1p and p40Sic1 (Nasmyth 1993). The degradation machinery for Far1p and p40Sic1, however, remains fully active throughout the G₂ and M phases of the cell cycle, as indicated by the G₁-specific presence of Far1p expressed from a constitutive promoter (Figs. 3C and 4). How is degradation maintained during phases at which Cdc28p–Cln1(2)p kinase activity is low? The stability of Far1-2p throughout the cell cycle indicates that degradation of Far1p in G₁ is still dependent on phosphorylation of Ser-87. We propose that in G₂, a kinase other than Cdc28p–Cln1(2)p—perhaps Cdc28p complexed with Cln3p or Clb1p cyclins—can phosphorylate Far1p on this site.

Far1p and Cdc28p–Cln1p kinase antagonize each other.

Far1p was identified because it is able to inhibit the activity of the Cdc28p–Cln1p kinase (Chang and Herskowitz 1990; Peter and Herskowitz 1994). Our results now show that Cdc28p–Cln1p, in turn, inhibits Far1p by triggering its degradation, thus establishing a negative feedback loop between Far1p and the Cdc28p–Cln1p kinase (Cross 1995; Peter 1997). Such a negative feedback loop might serve to amplify small differences between the activities of Far1p and Cdc28p–Cln1p kinase. It appears, therefore, that the balance between the activity of the G₁ kinase Cdc28p–Cln1p and its inhibitor Far1p determines whether a cell divides or arrests. The activity of the G₁ kinase Cdc28p–Cln1p sets a threshold that inhibitors such as Far1p must overcome to arrest cells at Start. In the absence of pheromones, Far1p is turned over rapidly by ubiquitin-dependent proteolysis, thereby preventing accumulation of Far1p to levels sufficient to inhibit Cdc28p–Cln1p and allowing cell cycle progression. The balance of Far1p relative to Cdc28p–Cln1(2)p can be tipped by production of a stable Far1p or by activation of the pheromone response pathway. The pheromone response pathway decreases the levels of the G₁-cyclins Cln1p and Cln2p (Wittenberg et al. 1990) and increases the levels of Far1p (Chang and Herskowitz 1990; Oehlen et al. 1996). Overexpression of the G₁-cyclin Cln2p, either by strong promoters or by stabilization of the protein, increases the threshold level set by the G₁ kinase, causing cells to arrest poorly in response to pheromones (Lanker et al. 1996). Cell cycle arrest can be fully restored by simultaneous overexpression of Far1p (S. Henchoz and M. Peter, unpublished).

Far1p levels are increased in response to mating pheromones by transcriptional activation of the FAR1 gene (Chang and Herskowitz 1990) and possibly by preventing Far1p degradation. Preliminary results indicate that the half-life of Far1p is increased after treating cells with α-factor by a mechanism that is dependent on the MAP kinase Fus3p (S. Henchoz and M. Peter, unpublished). It is interesting to note that other MAP kinase signal trans-
duction pathways might similarly regulate ubiquitin-dependent proteolysis. For example, activation of a MAP kinase-signaling cascade in Xenopus eggs prevents degradation of B-type cyclins, which leads to cell cycle arrest in metaphase of meiosis II (Minshull et al. 1994). Likewise, the spindle checkpoint activates a MAP kinase cascade resulting in cell cycle arrest by preventing the scheduled degradation of mitotic cyclins and possibly other destruction box substrates (Hardwick et al. 1996). Finally, the proto-oncogene c-jun is protected from ubiquitin-dependent degradation after phosphorylation by MAP kinases (Musi et al. 1997). It appears, therefore, that several signaling pathways might control the stability of downstream regulators by preventing their ubiquitin-dependent degradation.

Ubiquitin-dependent degradation: a general mechanism to regulate CKIs?

CKIs from other species might similarly be regulated by ubiquitin-dependent proteolysis (Peter 1997). The half-life of p27Kip1 increases upon contact inhibition of cells and decreases when HeLa cells are arrested in S phase (Hengst and Reed 1996). Consistent with these results, S phase extracts prepared from Swiss 3T3 cells degrade p27Kip1 in vitro whereas mid-G1 extracts do not (Brandes and Hunt 1996). Furthermore, p27Kip1 protein levels decrease when cells enter the cell cycle from quiescent state or in response to rapamycin (Nourse et al. 1994). Extracts prepared from proliferating cells ubiquitinate p27Kip1 protein at higher rates than extracts prepared from quiescent cells (Pagano et al. 1995). Recent studies indicate that the signal for ubiquitination and subsequent degradation of p27Kip1 might also be phosphorylation. Mutations that affect putative sites of phosphorylation by associated Cdk2-cyclin E kinase stabilize p27Kip1 in vivo (Sheaff et al. 1997; Vlach et al. 1997). Finally, increased proteosome-mediated degradation of p27Kip1 has been observed in certain tumors and correlates with poor prognosis (Loda et al. 1997), suggesting that the ubiquitin-dependent degradation pathway may be involved in tumorogenesis. Ubiquitin-dependent degradation triggered by phosphorylation may represent a conserved mechanism to regulate the activity of CKIs through the cell cycle and in response to extracellular signals.

Materials and methods

Yeast strains

Yeast strains are described in Table 1. Standard yeast growth conditions and genetic manipulations were used as described (Rose and Fink 1990). Yeast transformations were performed by the lithium acetate procedure (Ito et al. 1983).

DNA manipulations

Standard procedures were used for recombinant DNA manipulations (Ausubel et al. 1991; Sambrook et al. 1989). PCR was performed with Vent polymerase (New England Biolabs). PCR products were purified with the Wizard PCR purification kit according to the instructions of the manufacturer (Promega). Site directed mutagenesis was carried out by use of M13 mutagenesis as recommended by the manufacturer (Amersham). DNA sequencing was performed on both strands with the Sequenase kit (United States Biochemical Corp.).

Isolation of FAR1-22 (pTP63)

An amino-terminal fragment of FAR1 was mutagenized by PCR (Leung et al. 1989) by use of the primers oTP52 (5'-CGGCTGGATGAAGACACAACAAAGGTCGG-3') and oTP55 (5'-CCGACGGTTACCCATAACAGTTTACACAC-3') and plasmid pTP62 (pGAL–FAR1) as a template. The PCR product was digested with Xhol and KpnI, ligated into the vector pTP62, which had been digested with Xhol and KpnI, and transformed into DH5α. Transformants were pooled, and the isolated plasmid DNA transformed into the yeast strain K699. Individual yeast transformants were patched on SD–URA plates and subsequently replica-plated onto GAL–URA medium. Plasmids from transformants unable to grow on GAL–URA were isolated and the FAR1 coding sequence subcloned into Bluescript KS+ vector for sequencing. The Xhol–SphI fragment of FAR1-22 was ligated into the Xhol–SphI sites of pTP62 to yield plasmid pTP63.

Construction of integrating plasmids for Far1p and Far1-22p

For integration of wild-type FAR1 and FAR1-22 expressed from the GAL promoter, the entire FAR1 coding sequence with the GAL1 promoter was isolated from pTP62 and pTP63, respectively, after digestion with PvuII. The fragments were then ligated to PvuII cut pRS304 (Sikorski and Hieter 1989) to yield plasmid pTP69 (wild-type FAR1) or pTP70 (FAR1-22). The plasmids were digested with Bsu36I to target integration to the TRP1 locus.

Construction of epitope-tagged versions of Far1p

An EcoRI site was introduced by PCR at the stop codon of FAR1 by use of pTP62 as a template and primers oTP322 (5'-TGTGGATATTTGCTCGATGCTG-3') and oTP332 (5'-CGAGAATTCCGATGCTGAGG-3') and oTP323 (5'-CGAGAATTCCGATGCTGAGG-3'). pTP69 was digested with Xhol, ligated into the vector pTP62, and transformed into the yeast strain K699. Individual yeast transformants were pooled, and the isolated plasmid DNA transformed into the yeast strain K699. Individual yeast transformants were patched on SD–URA plates and subsequently replica-plated onto GAL–URA medium. Plasmids from transformants unable to grow on GAL–URA were isolated and the FAR1 coding sequence subcloned into Bluescript KS+ vector for sequencing. The Xhol–SphI fragment of FAR1-22 was ligated into the Xhol–SphI sites of pTP62 to yield plasmid pTP63.

DNA sequencing was performed on both strands with the Sequenase kit (United States Biochemical Corp.). Site directed mutagenesis was carried out by use of M13 mutagenesis as recommended by the manufacturer (Amersham). DNA sequencing was performed on both strands with the Sequenase kit (United States Biochemical Corp.).
monoclonal antibodies against GFP or with affinity-purified antibodies against Far1p (Peter et al. 1993) and developed by use of epichemiluminescence (Amersham Corp., Arlington Heights, IL).

Kinase assays and expression of GST-Far1p

GST-Far1 fusion proteins to be used as substrates in the kinase reactions were expressed in E. coli and purified as described (Peter and Herskowitz 1994a). Histone H1 was purchased from Boehringer Mannheim (No 1004 875). Cdc28p-Cln2p was immunoprecipitated from extracts as described. For in vitro phosphorylation assays, Cdc28p-Cln2p kinase was isolated from cells overexpressing Cln2-HAp or untagged Cln2p as a control from the constitutive ADH1 promoter (Peter and Herskowitz 1994a). Kinase assays were performed as described previously (Peter and Herskowitz 1994a).

Phosphoamino acid analysis was performed according to Peter et al. (1990). Briefly, phosphorylated proteins were eluted from SDS gels, hydrolyzed at 110°C for 1 hr in 6 M HCl, and the

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### Table 1. Yeast strains

| Strain   | Genotype | Source                      |
|----------|----------|-----------------------------|
| IH1792   | MATα cry1 lys1 | collection                  |
| IH1793   | MATα lys1    | collection                  |
| IH2518   | MATα ura3 leu2 trp1 his4 cyh2 bar1 | F. Cross (Rockefeller University, New York, NY) |
| IH2517   | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3, GAL*, psi*, ssd1-d2 | K. Nasmyth (IMP, Vienna, Austria) |
| K699     | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMP562   | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMP1055  | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMP126   | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMP127   | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| K4102    | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMT670   | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| ES464    | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMT668   | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMT740   | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMT263   | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| Y80      | MATα can1-100 ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 | S. Elledge (Baylor College of Medicine, Houston, TX) |
| Y552     | MATα can1-100 ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 skpl-11 | S. Elledge |
| Y554     | MATα can1-100 ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 skpl-12 | S. Elledge |
| EY957    | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | E. Elion (Harvard University, Boston, MA) |
| K2149    | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | G. Ammerer (University of Vienna, Austria) |
| K2180    | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | G. Ammerer |
| K2297    | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | G. Ammerer |
| YMP1054  | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMP1056  | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
| YMP1057  | MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL* psi* ssd1-d2 | this study |
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resulting mixture was separated in two dimensions on TLC plates.

Determination of half-life and quantification

Cells harboring plasmids encoding FAR1-GFP (pTP68) or FAR1-22-GFP (pTP91) were grown at 30°C to early log phase in raffinose-URA medium (2% raffinose), at which time expression was induced for 4 hr by addition of galactose (2% final concentration). A first aliquot was removed before expression was turned off by addition of glucose to a final concentration of 2%. Aliquots were collected every 30 min, and Far1p levels analyzed by immunoblotting. Western blots were quantified with the Image Quant™ software (Molecular Dynamics, Inc.). The half-life of Far1p was shifted to 37°C for 2 hr, at which time the GAL promoter was repressed by the addition of glucose (2% final). Aliquots were collected and analyzed as described above.

Determination of Far1-LacZ levels

Transformants containing either pBC1 (GAL–FAR1-lacZ) or pBC2 (GAL–FAR1-22-LacZ), were grown at 30°C in raffinose-LEU medium to early log phase at which time the temperature was shifted to 37°C to induce cell cycle arrest of thecdc mutants. After 90 min, the GAL promoter was induced by addition of galactose to 2% final concentration. Cells were harvested 2 hr later and β-galactosidase levels were determined as described (Stern et al. 1984), except that because of the different arrest morphology, Miller units were normalized to cell number rather than OD600. Miller units were determined from three independent experiments.

Microscopy and flow cytometry

Cells were grown as indicated, sonicated, fixed with formaldehyde to a final concentration of 3.7%, and viewed by differential interference contrast microscopy. Yeast actin was visualized by rhodamin phalloidin (Molecular Probes Inc., Eugene, OR) as described previously (Peter et al. 1996).

Cells harboring plasmids encoding Far1p-GFP (pTP68) or Far1-22p-GFP (pTP91) expressed from the GAL promoter were grown in raffinose-URA medium (2% raffinose) at 30°C. Expression was induced for 4 hr by addition of galactose to 2% final concentration. GFP fluorescence was visualized with an argon laser at 488 nm with a Zeiss Axiosvert 100 microscope (Zeiss laser Scanning Microscope 410) with a 63× Plan-ApoChromat objective (1.4 oil). Standardized conditions for the pinhole size, gain, and offset (brightness and contrast) were used for image capture. Each image was the average of eight scans. Image capture and background subtraction were performed to allow direct comparisons.

Flow cytometry was carried out as described in Epstein and Cross (1992). Briefly, cells were fixed in 70% ethanol, washed and digested with RNase A for 5 hr. The DNA was stained then with propidium iodine and analyzed on a FACScan (Becton-Dickinson).

Ubiquitination assays

A cell-free in vitro ubiquitination assay system in budding yeast has been described previously (Verma et al. 1997a,c). To summarize, both wild-type and mutant FAR1 (Fig. 8A) transcription templates were generated from pTP62 and pTP63, respectively, by PCR by use of a 5′ oligonucleotide containing a T7 RNA polymerase promoter (Verma et al. 1997a). The 5′ oligonucleotide is (GCCGAGCTCTAATACGACTCACTATAGGTCATTCTTAACGTCAAGGAG) and the 3′ oligonucleotide is (GGCGGATCCCTAGGGGTGGAACTTCCAG). Alternatively, a linearized PET24b plasmid carrying T7-directed wild-type FAR1 with a carboxy-terminal His tag (provided by F. Cross) was used (Fig. 8B). The templates were then transcribed and translated in vitro according to the manufacturers instructions (Promega) to generate [35S]methionine-labeled Far1p substrates. Both wild-type (Fig. 8A) and cdc4 (Fig. 8B) crude extracts were made from G1 cyclin-depleted cells and then fractionated on DEAE Sepharose columns. The 0.25 M NaCl eluate, which lacked the endogenous Cdc34p, was used in these assays. Cdc34p was purified from E. coli as described (Banerjee et al. 1993). Cdc4p and Cdc28p were expressed in baculovirus-infected insect cells (Verma et al. 1997c). The radiolabeled substrate was incubated in 10 µl of reaction mixture (Verma et al. 1997a) containing yeast extract (100 µg), reaction buffer containing protease inhibitors, ATP mix, ubiquitin (10 µg), and Cdc34p (100 ng). For reactions with cdc4, 0.5 µl of insect lysate (−5 mg/ml) containing Cdc4p or Cdc28p was used. Reactions were incubated at 24°C for 60 min, terminated by the addition of SDS-PAGE sample buffer, boiled, and evaluated by SDS-PAGE and autoradiography.

Pheromone response and mating assays

Halo and mating assays were carried out as described previously (Valtz and Peter 1997). To measure induction of the FU1–LacZ construct, cells (YM1055) producing either wild-type Far1p or Far1-22p from the inducible GAL promoter for 6 hr were exposed to α-factor (1 µg/ml) for 1 hr. β-galactosidase levels were quantified as described (Stern et al. 1984). For the α-factor release experiments, strain K2180 producing either wild-type Far1p or Far1-22p from the GAL promoter was grown in raffinose-URA medium (2% raffinose) at 30°C, at which time expression of Far1p and cell cycle arrest was induced by addition of galactose (2% final concentration) and α-factor (1 µg/ml). After 3 hr, cells were washed twice with prewarmed GAL–URA medium (2% galactose) and inoculated in GAL–URA medium at 30°C (time 0). Samples were removed every 30 min and protein extracts prepared as described. An aliquot of cells was fixed, sonicated and the percentage of unbudded cells was determined microscopically as described (Valtz and Peter 1997).

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