

Degradation of the Transcription Factor Gcn4 Requires the Kinase Pho85 and the SCF^{CDC4} Ubiquitin–Ligase Complex

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Gcn4, a yeast transcriptional activator that promotes the expression of amino acid and purine biosynthesis genes, is rapidly degraded in rich medium. Here we report that SCF^{CDC4}, a recently characterized protein complex that acts in conjunction with the ubiquitin-conjugating enzyme Cdc34 to degrade cell cycle regulators, is also necessary for the degradation of the transcription factor Gcn4. Degradation of Gcn4 occurs throughout the cell cycle, whereas degradation of the known cell cycle substrates of Cdc34/SCF^{CDC4} is cell cycle regulated. Gcn4 ubiquitination and degradation are regulated by starvation for amino acids, whereas the degradation of the cell cycle substrates of Cdc34/SCF^{CDC4} is unaffected by starvation. We further show that unlike the cell cycle substrates of Cdc34/SCF^{CDC4}, which require phosphorylation by the kinase Cdc28, Gcn4 degradation requires the kinase Pho85. We identify the critical target site of Pho85 on Gcn4; a mutation of this site stabilizes the protein. A specific Pho85-Pcl complex that is able to phosphorylate Gcn4 on that site is inactive under conditions under which Gcn4 is stable. Thus, Cdc34/SCF^{CDC4} activity is constitutive, and regulation of the stability of its various substrates occurs at the level of their phosphorylation.

INTRODUCTION

Regulation of cellular processes occurs in a large measure via modulation of steady-state levels of key regulatory proteins. This modulation can occur not only at the level of their synthesis but also at the level of their degradation. Gcn4, a yeast transcriptional activator involved in biosynthesis of amino acids and purines (Hinnebusch and Fink, 1983; Hope and Struhl, 1985), is under this dual control. Starvation for amino acids leads to an increase in Gcn4 translation (Hinnebusch, 1984), which in turn induces the transcription of amino acid biosynthetic genes, thereby providing the nutrients that were formerly limiting for growth. The levels of Gcn4 protein are controlled both at the level of synthesis and degradation. Gcn4 synthesis is increased by a translational control mechanism that involves phosphorylation of the general translation initiation factor eIF-2 α by the kinase Gcn2 (Dever *et al.*, 1992; for review, see Hinnebusch, 1997). In addition, Gcn4 is degraded extremely rapidly; however,

under amino acid starvation conditions, Gcn4 is stabilized (Kornitzer *et al.*, 1994).

The ubiquitin system is the major cytoplasmic pathway by which proteins are degraded (for a recent review, see Hershko and Ciechanover, 1998). Target proteins are first modified by the sequential addition of ubiquitin, a small, conserved protein. Ubiquitinated proteins are then degraded by a large multicatalytic protease, the 26S proteasome. Protein ubiquitination is catalyzed by a ubiquitin-conjugating enzyme, which accepts a ubiquitin “activated” as a thiolester by the ubiquitin-activating enzyme, and transfers it to the ϵ -amino group of an internal lysine on the target protein (or to the α -amino group of the polypeptide chain; Breitschopf *et al.*, 1998). The transfer from the ubiquitin-conjugating enzyme to the target requires a third component, called ubiquitin ligase, which constitutes the main recognition component of the system and in some cases may form a second intermediate thiolester with ubiquitin.

Unlike the ubiquitin-conjugating enzymes, which carry a distinctive sequence signature, ubiquitin ligases fall in a number of groups with little sequence similarity. At least four types of ligases can be distinguished, two of which consist of large protein complexes (Hershko and Ciech-

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anover, 1998). These are the anaphase-promoting complex (APC) or cyclosome (King *et al.*, 1995; Sudakin *et al.*, 1995) and the SCF complex (Feldman *et al.*, 1997; Skowryra *et al.*, 1997). The latter stands for Skp1-Cdc53 (or Cullin)-F box protein complex, in which the F-box protein constitutes a variable component that is thought to define the target specificity of the complex (for review, see Patton *et al.*, 1998b). A fourth component of the SCF complex, Rbx1, recently was discovered (Kamura *et al.*, 1999; Skowryra *et al.*, 1999). The SCF ubiquitin ligase is found to interact with a single type of ubiquitin-conjugating enzyme, Cdc34 (Feldman *et al.*, 1997; Skowryra *et al.*, 1997; Mathias *et al.*, 1998). Targets of the SCF^{CDC4} complex (the SCF complex containing Cdc4 as the F-box component) in yeast that have been identified so far include the cyclin-dependent kinase (CDK) inhibitors Sic1 (Feldman *et al.*, 1997) and Far1 (Henchoz *et al.*, 1997) and the DNA replication regulator Cdc6 (Drury *et al.*, 1997; Elsasser *et al.*, 1999; Sanchez *et al.*, 1999). Other yeast SCF complexes were found to target other substrates, such as Cln1 and -2 and Gic2 (SCF^{GRR1}; Skowryra *et al.*, 1997; Jaquenoud *et al.*, 1998; Patton *et al.*, 1998a) and the inhibitory kinase Swe1 (SCF^{MET30}; Kaiser *et al.*, 1998). In mammalian cells, a specific SCF complex, SCF ^{β -TrCP}, recently was found to target I κ B for ubiquitination (Yaron *et al.*, 1998; Spencer *et al.*, 1999; Winston *et al.*, 1999). Unlike APC substrates, which contain a consensus sequence required for degradation (the "destruction box"; Yamano *et al.*, 1996), no such consensus has been found yet for the SCF substrates. However, common to many, and perhaps all, instances of ubiquitination by SCF complexes is the requirement for phosphorylation of the substrate (Hershko and Ciechanover, 1998). In yeast, ubiquitination of the CDK inhibitors Sic1 and Far1 and of the DNA replication regulator Cdc6 were shown to require activity of the major yeast cell cycle CDK, Cdc28 (Henchoz *et al.*, 1997; Verma *et al.*, 1997; Elsasser *et al.*, 1999). A second yeast CDK, Pho85, which is not essential for cell cycle progression, recently was found to be also capable of phosphorylating Sic1 (Nishizawa *et al.*, 1998).

Previous work has shown that Gcn4 is degraded via the ubiquitin system and stabilized in proteasome mutants, as well as in a mutant of the ubiquitin-conjugating enzyme Cdc34 (Kornitzer *et al.*, 1994). However, neither the precise ubiquitination complex required for Gcn4 degradation nor the mechanism of regulation of Gcn4 degradation by starvation had been defined. Here we show that degradation of Gcn4 requires the kinase Pho85 and the SCF^{CDC4} ubiquitin-ligase complex. Our data indicate that Pho85 activity is repressed by starvation and suggest that the targeting of Gcn4 to the ubiquitin-ligase complex SCF^{CDC4} is determined by a specific Pho85-mediated phosphorylation event.

MATERIALS AND METHODS

Plasmids and Strains

The *CUP1* vector plasmid KB354 was constructed by cloning the *Bam*HI-*Eco*RI *CUP1* promoter fragment from plasmid pYSK7 (Butt *et al.*, 1984) to pRS314 (Sikorski and Hieter, 1989), followed by deletion of the *Sac*II-*Bam*HI sequence of the polylinker. PCR cloning (Ausubel *et al.*, 1989) was used to fuse the coding sequence of *GCN4* to the *CUP1* promoter of KB354. The resulting plasmid was then digested with *Bam*HI and *Hind*III to generate a fragment carrying part of the *TRP1* sequence, the *CUP1* promoter sequence, and the 5' end of the *GCN4* sequence. This fragment was cloned in KB64

digested with the same enzymes, to generate a *CUP1p-GCN4-LacZ* fusion (KB449). *pGAL-CYC1-LacZ* (KB496) was constructed by inserting a PCR-generated *Bgl*III fragment carrying the whole *GCN4* coding sequence into the *Bam*HI site of plasmid pLGSD5 (from L. Guarente, Massachusetts Institute of Technology). *pGAL-CDC6-LacZ* (KB448) was constructed by substituting the *GCN4* coding region of plasmid KB64 (Kornitzer *et al.*, 1994) with a PCR-generated *Bam*HI-*Eco*RI fragment carrying the *CDC6* coding sequence. The T165A mutation was introduced by site-directed mutagenesis into the *ADE8-GCN4* construct KB105 (Kornitzer *et al.*, 1994), to generate KB853. PCR cloning was used to subsequently transfer the mutation to the *pGAL-GCN4-LacZ* plasmid KB149, to generate KB854. The *pCUP1-SIC1* plasmid (KB733) was generated by PCR cloning of an *Eco*RI-*Sall* fragment carrying *SIC1* into the *pCUP1* vector KB354 digested with the same enzymes, to generate KB733. *pGAL1-CLN2(4T3S)HA* expressing a stabilized version of Cln2 (Lanker *et al.*, 1996) was obtained from Curt Wittenberg (Scripps Research Institute). The *GCN4* deletion $\Delta(93-140)$ (KB 92) was described previously (Kornitzer *et al.*, 1994). The *GCN4* deletion $\Delta(151-167)$ fused to *LacZ* (KB295) was generated by digestion of the *GCN4* coding sequence with *Kpn*I and *Xba*I, filling-in, and religation. The plasmid expressing the myc-tagged Gcn4(62-202) fragment was constructed in two steps: first, PCR was used to generate an *Eco*RI site and an initiation codon in front of the Gcn4(62-202)-encoding fragment and an *Xho*I site preceded by a termination codon and a *Bam*HI site at the other end of the fragment (forward primer: 5' CCGAATTCATGTCGAACCTTGATTTTGATT; reverse primer: 5' GGCCTCGAGTTAGGATCCCATATGATCCAGTCTCGATTTCG). This fragment was then cloned in the *Eco*RI and *Xho*I sites of plasmid KB354 to generate KB 885 (wild type) and KB 887 (T165A). In a second step, a triple Myc-epitope-carrying *Bam*HI fragment (Kornitzer and Kron, unpublished results) was cloned into the *Bam*HI site of plasmids KB885 and KB887 to generate KB891 and KB893, respectively. The *GST-Gcn4(62-202)*-expressing plasmid was constructed by recloning the *Eco*RI-*Xho*I fragments of KB885 and KB887 into pGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ) to generate KB927 and KB928, respectively. The plasmid expressing heptahistidine-tagged Gcn4 under the T7 promoter was constructed by cloning a Gcn4-carrying PCR fragment into pET11-his7 (obtained from S. Buratowski, Harvard Medical School) to generate KB363. Plasmids pGEX-PHO85, pGEX-pho85(E53A), pGEX-PCL1, pGEX-PHO80, and Yep-GAL10-HA-PCL1 are described in Nishizawa *et al.* (1998).

The *cdc4-1* strain (KY301) was constructed by homologous recombination. The region extending from -270 to +2575 relative to the *CDC4* start site was cloned by PCR from a *cdc4-1* strain (Fink laboratory collection) between the *Bam*HI and *Eco*RI sites of plasmid Yip5, to yield KB527. Strain KY289 (an α strain isogenic to KY204) was transformed with *Kpn*I-digested KB527, to yield a tandem mutant-wild-type structure in the chromosome, separated by the Yip5 sequence. Passage over 5-fluoro-orotic acid to select for excision of the *URA3* sequence of Yip5 by homologous recombination yielded a fraction of colonies in which the wild-type allele was excised and the mutant retained in the chromosome. The *pho85 Δ ::hisG* strain (DY4535) was constructed in several steps. First, the *PHO85* gene in a diploid W303 strain was replaced with a *pho85 Δ ::LEU2* allele using a disruption cassette provided by Brenda Andrews (University of Toronto). A *pho85 Δ ::LEU2* haploid strain was derived from this diploid, and this was converted to a *pho85 Δ ::hisG-URA3-hisG* allele using plasmid pNKY85 (Alani *et al.*, 1987). Finally, the strain with the *pho85 Δ ::hisG* allele was recovered after passage over 5-fluoro-orotic acid medium. The other yeast strains used and their origins are described in Table 1.

Degradation Assays

The pulse-chase experiments were performed essentially as described previously (Kornitzer *et al.*, 1994). Briefly, overnight cultures were diluted in 10 ml and grown to midlog phase, washed two

Table 1. Yeast strains

Name	Genotype	Source
KY26	a <i>ura3-52 leu2-Δ2 trp1-Δ1 bas1-2 bas2-2 gcn4-Δ1</i>	Kornitzer <i>et al.</i> (1994)
KY204	KY26 <i>ade8-GCN4</i>	Kornitzer <i>et al.</i> (1994)
KY205	KY204 <i>cdc34-2</i>	Kornitzer <i>et al.</i> (1994)
KY301	KY204 <i>cdc4-1</i>	This work
W303-1A	<i>ura3-1 ade2-1 can1-100 leu2-3,112 trp1-1his3-11,15</i>	R. Rothstein
DY4535	W303 <i>pho85Δ::hisG</i>	This work
Y552	W303 <i>skp1-11</i>	Bai <i>et al.</i> (1996)
Y554	W303 <i>skp1-12</i>	Bai <i>et al.</i> (1996)
YPH1015	<i>ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i> <i>CFIII(CEN3.L.YPH983)-HIS3-SUP11</i>	Connelly and Hieter (1996)
YPH1161	YPH1015 <i>LEU2::skp1-4</i>	Connelly and Hieter (1996)
YPH1172	YPH1015 <i>LEU2::skp1-3</i>	Connelly and Hieter (1996)
MTY668	W303 <i>cdc4-1</i>	M. Tyers
MTY670	W303 <i>cdc34-2</i>	M. Tyers
MTY740	W303 <i>cdc53-1</i>	Willems <i>et al.</i> (1996)

times with medium lacking methionine, concentrated to 0.3 ml, and pulse-labeled for 5 min with 750–900 mCi [³⁵S]methionine (“express,” NEN, Arlington, MA), pelleted again and chased in medium containing 10 mM methionine and 10 mM cysteine. For the experiments involving temperature-sensitive mutants, the cells were shifted from 30 to 37°C 20–40 min before labeling. Similarly, for the starvation studies, cells were harvested by centrifugation and grown in SD medium + adenine for 15–45 min before labeling and chased in the same medium + methionine and cysteine. At various times of the chase, an aliquot of the culture was removed and incubated 15 min on ice with 0.35 M NaOH and 1.5% 2-mercaptoethanol, followed by precipitation with 6% trichloroacetic acid. The protein precipitate was resuspended by boiling in 2.5% SDS and 5 mM EDTA, and equal amounts of trichloroacetic acid-precipitable radioactivity were immunoprecipitated in at least 10 volumes of buffer A (Hochstrasser and Varshavsky, 1990) containing protein A-Sepharose (Pharmacia). The immunoprecipitates were run on SDS-polyacrylamide gels, and the protein bands were quantitated using a Fujix Bas 2000 bioimage analyzer (Fuji, Tokyo, Japan). Antibodies used were either monoclonal anti-myc (9E10; Kolodziej and Young, 1991), anti-β-galactosidase (Cappel, Malvern, PA), or anti-Gcn4, which were generated in rabbits injected with recombinant GST-Gcn4 fusion protein. The proteins we tested were usually ectopically expressed either from the *GAL1* promoter or from the *CUP1* promoter. *CUP1* is induced by copper ions; however, even in the absence of copper, enough protein is produced to permit detection in pulse-labeling assays. The half-life of Gcn4 expressed from the *CUP1* promoter (Figures 1, 2A, 3, and 7A), 2–3 min, was somewhat shorter than that of the same protein expressed under the *GAL1* promoter (3–5 min; see Kornitzer *et al.* [1994] and Figures 2D, 6, and 8). This difference may be due to the difference in the carbon source used (glucose vs. galactose). For the experiments performed in cell cycle-arrested cells, 3 μM α-factor or 0.2 M hydroxyurea were used to arrest cells in G1 and S, respectively. Cell cycle arrest was monitored microscopically; the labeling was initiated after >90% of the cells had arrested as unbudded or large-budded cells, respectively.

In Vivo Phosphorylation Assay

For in vivo phosphate labeling, cells were grown overnight in yeast nitrogen base (YNB) with the required amino acids and a reduced KH₂PO₄ concentration (1 instead of 6 mM). The cells were then diluted in the same medium but with 0.1 mM KH₂PO₄, grown to optical density (OD)₆₀₀ = 0.5, and treated with 1 μg/ml cycloheximide or transferred to YNB devoid of the required amino acids to

induce starvation, as required. Cells were then washed twice with the same medium containing only 50 μM KH₂PO₄, and each sample (5 OD units of cells) was labeled for 15 min in 0.5 ml of the same medium containing 1 mCi ³²P₄. Cell extract preparation and immunoprecipitation were performed as for the degradation assay.

Kinase Assays

For the in vitro kinase reactions with recombinant proteins, 10 ng GST-PHO85, 10 ng GST-cyclin, and 0.5 μg substrate were incubated at 30°C for 30 min with 1 μCi [³²P-γ]ATP and 0.1 mM cold ATP in 10 μl kinase assay buffer (50 mM Tris, 10 mM MgCl₂, 2 mM EDTA, 1 mM dithiothreitol, pH 7.5). Recombinant proteins were expressed and purified according to standard protocols. An ultrafiltration centrifugal device was used for buffer exchange and concentration of the protein. For the immunoprecipitated kinase assays, extracts of 50 ml exponentially growing culture induced 4 h with galactose to express ha-Pcl1 and starved or cycloheximide-treated as indicated were made by breaking the cells for 5 min with glass beads in extract buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% NP40, phosphatase inhibitors: 2 mM Na-pyrophosphate, 80 mM β-glycerolphosphate, 10 mM NaF, 0.3 mM NaVO₃, protease inhibitors: 2 mM benzamide, 1 mM phenylmethylsulfonyl fluoride, aprotinin 1:100, TPCK 50 μg/ml, TLCK 50 μg/ml, and 1:500 of an antiprotease cocktail containing leupeptin, pepstatin, and chymostatin, each 10 mg/ml in dimethyl sulfoxide). Five to 20 μl of 0.2 mg protein extract were incubated with the 12CA5 monoclonal antibody for 30 min on ice and then 20 μl of a 50% slurry of protein A-agarose beads (Pharmacia) and 200 μl extract buffer were added, and the tubes were incubating while tumbling for 1 h at 4°C. The agarose beads were then washed three times with extract buffer lacking aprotinin, TPCK, TLCK, and the 500× antiprotease cocktail, and two times with kinase buffer. For each phosphorylation reaction, the agarose bead pellet was incubated 20 min at 30°C with 0.2 μg substrate in 10 μl kinase buffer with 1 μCi [³²P-γ]ATP and 20 μM cold ATP. All the kinase reactions were terminated by the addition of 10 μl protein-loading buffer and electrophoresed on an SDS-PAGE gel.

RESULTS

Inhibition of Translation Leads to Gcn4 Stabilization

Degradation of Gcn4 occurs through the ubiquitin pathway and is inhibited under amino acid starvation conditions or in

an amino acyl-tRNA synthetase mutant (Kornitzer *et al.*, 1994). To define further the signal that inhibits Gcn4 degradation, we examined the role of translation on Gcn4 degradation. In previous pulse-chase experiments designed to measure the rate of Gcn4 decay, we noticed that the addition of the translational inhibitor cycloheximide at the beginning of the chase typically reduced the rate of Gcn4 decay starting 10 min into the chase (see Kornitzer *et al.*, 1994). To test the possibility that inhibition of protein synthesis can reduce Gcn4 turnover, Gcn4-expressing cells were preincubated with 0.5 $\mu\text{g}/\text{ml}$ cycloheximide, so as to reduce protein synthesis by 80–90%. This pretreatment with the translation inhibitor led to a complete stabilization of the protein (Figure 1). In a separate experiment, cycloheximide was added to 0.1 $\mu\text{g}/\text{ml}$ or the translational inhibitor paromomycin was added to 10 mg/ml; under these conditions, translation was inhibited twofold at most, and the half-life of Gcn4 was increased twofold (our unpublished results).

Several SCF Components Are Implicated in Gcn4 Degradation

To clarify the regulatory mechanism of Gcn4 degradation, we first attempted to identify the ubiquitin ligase required for Gcn4 degradation. It was previously shown that Gcn4 is stabilized in a *cdc34* mutant (Kornitzer *et al.*, 1994). We asked

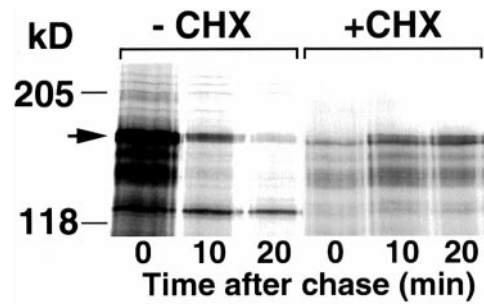


Figure 1. Inhibition of translation stabilizes Gcn4. Gcn4–LacZ was expressed from the *CUP1* promoter of plasmid KB449. +CHX: cells were preincubated for 30 min in 0.5 $\mu\text{g}/\text{ml}$ cycloheximide and then labeled and chased in the presence of the drug. The arrow on the left indicates Gcn4–LacZ.

whether mutants of the SCF complex would affect Gcn4 degradation. A Gcn4–LacZ fusion, previously shown to be degraded with the same kinetics as native Gcn4 (Kornitzer *et al.*, 1994), was placed under the control of the *CUP1* promoter (see MATERIALS AND METHODS). The *cdc4-1* mutant displays a cell cycle arrest phenotype similar to that of *cdc34-2*: both arrest at the G1 to S transition, with a mul-

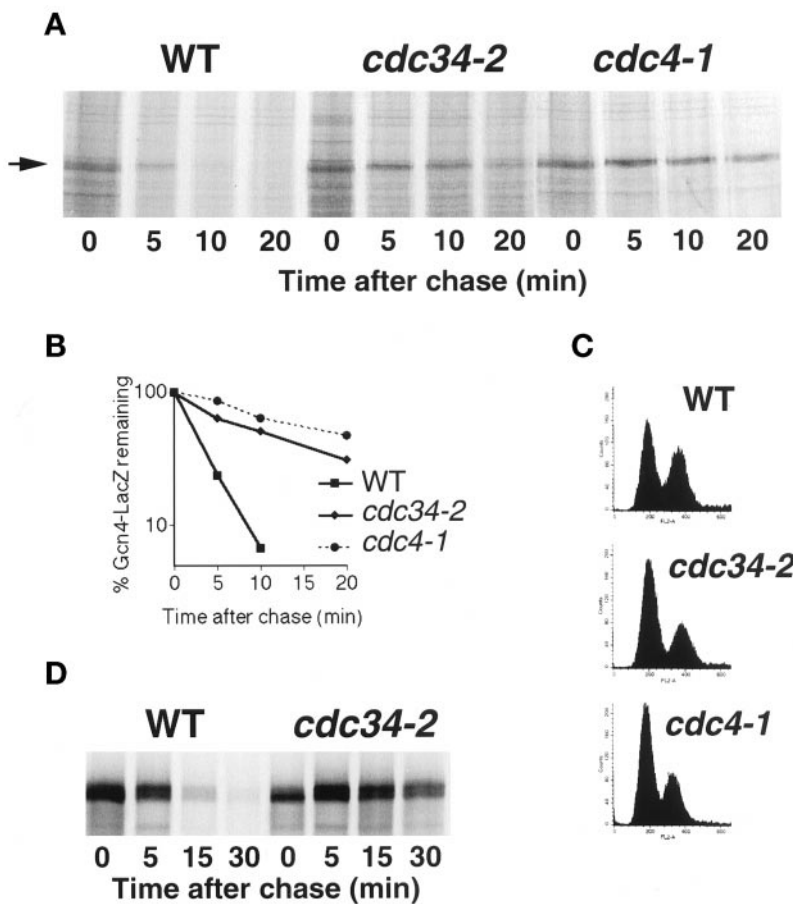


Figure 2. Gcn4 degradation is inhibited in *cdc34* and *cdc4* mutants. (A) Pulse-and-chase analysis of Gcn4–LacZ expressed from the *CUP1* promoter of plasmid KB449 in strains KY204, KY205, and KY301. The cultures were shifted from 30 to 37°C, 20 min before the labeling. (B) Quantitation of the experiment shown in (A). (C) Fluorescence-activated cell sorter analysis of cells at the time of labeling. (D) Pulse-chase analysis of Gcn4–LacZ expressed from the *GAL1* promoter of plasmid KB64 in α -factor-arrested cells. Cells were arrested with α -factor at the permissive temperature and then shifted to 37°C for 30 min before labeling.

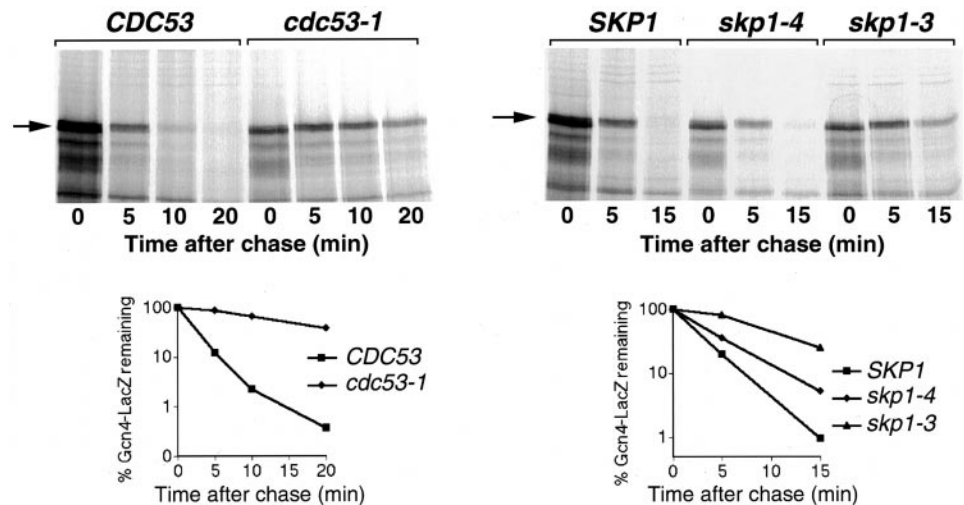


Figure 3. Gcn4 degradation is inhibited in *cdc53* and *skp1* mutants. Pulse-chase analysis of Gcn4–LacZ expressed from the *CUP1* promoter of plasmid KB449 in strains W303 and MTY740 (left) or YPH1015, YPH1161, and YPH1172 (right). The cultures were shifted from 30 to 37°C, 30 min before labeling.

tibudded phenotype (Yochem and Byers, 1987). We tested degradation of Gcn4 in *cdc34-2* and *cdc4-1* cells 20 min after they were shifted up to the nonpermissive temperature of 37°C. As can be seen in Figure 2, the half-life of Gcn4 is extended from 2.5 min to 12 and 20 min, respectively. We also tested whether the F-box protein Grr1, which is required for the degradation of G1 cyclins (Barral *et al.*, 1995; Skowyra *et al.*, 1997), is involved in Gcn4 degradation. No effect was seen on the half-life of Gcn4 in strains carrying a null allele of *GRR1* (our unpublished results).

One concern is that the stabilization of Gcn4 in the *cdc34* and *cdc4* mutants may be an indirect consequence of their cell cycle arrest rather than a direct consequence of their function in the ubiquitin pathway. However, if Gcn4 degradation were cell cycle dependent, then it should be biphasic in a population of cycling cells. Because we find that in cycling cells, Gcn4 degradation is exponential over two orders of magnitude (e.g., see Figures 2 and 3), if there is a cell cycle window where Gcn4 is stable, it has to be extremely narrow. It is still possible in theory that the *cdc34* and *cdc4* mutants arrest in that window. To address this concern, we analyzed the cell cycle distribution of the various strains at the time of labeling. Although the mutant strains do display a higher proportion of G1 cells than the wild-type strains (see Figure 2C), fully one-third of the mutant cells were still in G2/M at the time of labeling. Thus, the fact that cells in different stages of the cell cycle show stabilization of Gcn4 argues against the stabilization being an indirect effect of cell cycle arrest. This possibility was further addressed in an alternative way: wild-type and *cdc34* mutant cells expressing Gcn4–LacZ under the *GAL1,10* promoter were arrested in G1 with α -factor, the cells were shifted to the nonpermissive temperature, and Gcn4 degradation was assayed. As shown in Figure 2D, the pattern of degradation was identical to that in cycling cells (rapid in the wild type, very slow in the *cdc34* mutant), demonstrating that the stabilization of Gcn4 is unrelated to the cell cycle phenotype of *cdc34*.

Two other SCF components, Cdc53 and Skp1, were tested for effects on Gcn4 degradation. The *cdc53-1* mutant arrests with the same cell cycle phenotype as *cdc4* and *cdc34* (Mathias *et al.*, 1996). Pulse-chase analysis showed that

Gcn4 is strongly stabilized in *cdc53-1*, with an increase in the half-life of the protein from 2 to 15 min (Figure 3). *SKP1* was isolated as a suppressor of *cdc4-1* (Bai *et al.*, 1996), and various *skp1* alleles arrest in either G1 or G2/M of the cell cycle (Bai *et al.*, 1996; Connelly and Hieter, 1996). We found that, although the G1-arresting alleles *skp1-3* (Figure 3) and *skp1-11* (our unpublished results) strongly stabilized Gcn4, the effect of the G2-arresting alleles *skp1-4* (Figure 3) and *skp1-12* (our unpublished results) was more moderate.

We next tested the effects of overexpression of Gcn4 in wild-type versus mutant cells. Overexpression of Gcn4 leads to slower growth of wild-type cells but does not involve an obvious cell cycle delay (Kornitzer and Fink, unpublished observations). We reasoned that, if Gcn4 is a direct substrate of the Cdc34/SCF^{CDC4} complex, in mutant cells, where Cdc34/SCF^{CDC4} activity is limiting, Gcn4 overexpression may compete with the cell cycle substrates and lead to cell cycle arrest even at the normally permissive temperature. To test this, a Gcn4 construct under the strong galactose-inducible *GAL1*–*CYC1* hybrid promoter was expressed in wild-type and mutant cells. As can be seen in Figure 4, at the normally permissive temperature of 30°C, the presence of the Gcn4-overexpressing plasmid led to complete growth arrest of the *cdc34-2* and *cdc53-1* mutants on galactose medium and to strong growth repression of the *cdc4-1* mutant. Microscopic observation of these cells showed the characteristic multibudded phenotype of this class of cell division cycle mutants (our unpublished observations). The fact that overexpression of Gcn4 in these mutant backgrounds results in a mutant phenotype at 30° that mimics the phenotype at 37° supports the assumption that Gcn4 is a direct substrate of Cdc34/SCF^{CDC4} and that its overexpression competitively inhibits the complex.

Starvation Does Not Affect Degradation of Other SCF Substrates

Is Gcn4 ubiquitination regulated by modulation of Cdc34/SCF^{CDC4} activity or by modification of the substrate, Gcn4? If the Cdc34/SCF^{CDC4} complex were regulated, one would predict that amino acid starvation would stabilize other

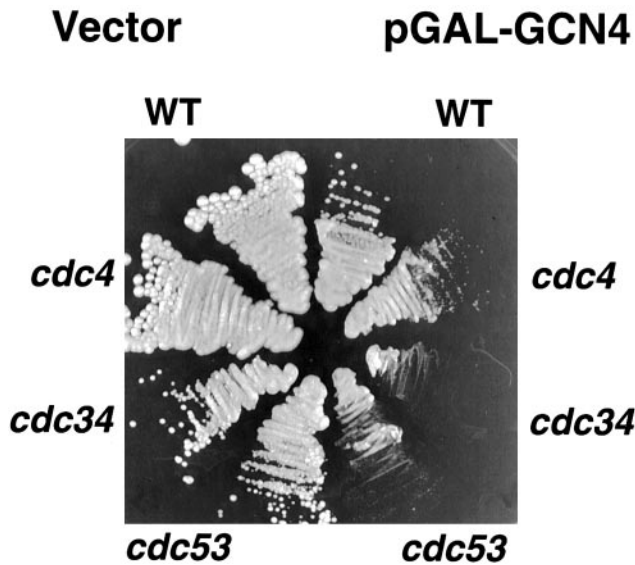


Figure 4. Effect of Gcn4 overexpression on the growth of various SCF mutants. Plasmid KB496, expressing Gcn4 under the strong GAL1–CYC1 hybrid promoter, or the vector plasmid pLGSD5, were transformed into the indicated strains. The strains were streaked on selective galactose plates and incubated for 3 d at 30°C.

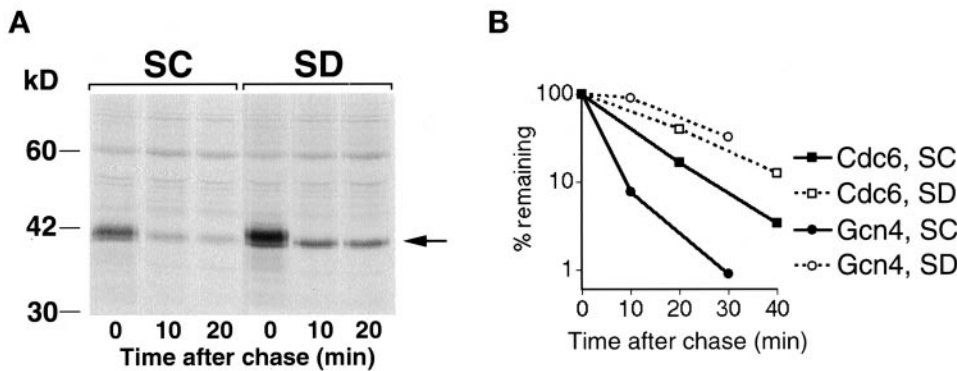
substrates of this complex. To test this, two additional substrates of the Cdc34/SCF^{CDC4} complex, the CDK inhibitor Sic1 and Cdc6, a protein involved in initiation of DNA replication, were analyzed for their stability. Sic1 degradation usually occurs at the end of G1 and is dependent upon phosphorylation by Cln1 or -2/Cdc28 (Schneider *et al.*, 1996; Tyers, 1996; Verma *et al.*, 1997). To dissociate the effects of starvation on Sic1 degradation from effects on the cell cycle progression, degradation was tested in S-phase-arrested cells in which a stable version of Cln2 (Lanker *et al.*, 1996) was ectopically expressed from the GAL1 promoter. Sic1 was constitutively expressed from the *CUP1* promoter. Un-

der these conditions, in rich medium, 90% of Sic1 was degraded in the first 10 min of the chase. The residual fraction appeared to be stable (Figure 5A) and could represent the Sic1 protein from a subpopulation of cells having lost the toxic GAL–CLN2 plasmid. Cells starved for amino acids display the same kinetics of Sic1 degradation, indicating that starvation does not affect the ubiquitination of this protein by the complex.

Cdc6 degradation is also cell cycle regulated and peaks in late G1/early S (Drury *et al.*, 1997). To measure Cdc6 degradation, a Cdc6–LacZ fusion was placed under the control of the GAL promoter, and degradation of the fusion protein was assayed in cells arrested in G1 with α -factor. The half-life of the Cdc6–lacZ fusion protein was 8 min in standard synthetic medium; in cells shifted to starvation medium 30 min before the chase, the half-life increased approximately twofold, to 15 min (Figure 5B). By comparison, in a parallel experiment, using the same starvation regimen, the half-life of Gcn4 increased from 3 to 21 min, i.e., sevenfold (Figure 5B). Thus, these results suggest that SCF^{CDC4} activity is not regulated by amino acid starvation.

Identification of a Sequence in Gcn4 Responsive to the Starvation Signal

If SCF^{CDC4} activity is constitutive, then the regulation of Gcn4 degradation must occur at the level of substrate modification. To identify the sequence in Gcn4 that is the target of this regulation, we tested a series of GCN4 deletions for degradation under starvation versus nonstarvation conditions. Previous deletion analysis identified an extended region of Gcn4, including at least residues 93–200, that is required for degradation (Kornitzer *et al.*, 1994). Finer deletion mapping allowed us to subdivide that region in at least two domains, extending from 93 to 118 (“I”) and from 140 to 200 (“II”). This subdivision is based on the observation that a deletion of residues 118–140 barely affects stability of the protein, whereas deletions N-terminal or C-terminal to these residues (e.g., Δ 151–167) significantly stabilize the protein (Kornitzer *et al.*, 1994; our unpublished results; Figure 6).



(pGAL1–CDC6–LacZ) or KB64 (pGAL1–GCN4–LacZ) were grown in galactose, arrested in G1 with α -factor, and subjected to pulse–chase analysis without (SC) or with (SD) prior amino acid starvation (30 min in medium lacking amino acids). The lacZ fusion proteins were visualized by immunoprecipitation with a specific LacZ antibody. The graph depicts the quantitation of the fusion protein band by phosphorimager.

Figure 5. Starvation does not affect degradation of two other SCF^{CDC4} substrates. (A) Degradation of Sic1. Cells carrying plasmids KB733 (*CUP1-SIC1*) and pGAL1–CLN2(4T3S)HA (Lanker *et al.*, 1996) were arrested in S-phase with hydroxyurea, induced with galactose to produce Cln2, and subjected to pulse–chase analysis without (SC) or with (SD) prior amino acid starvation. The Sic1 protein was visualized by immunoprecipitation with a specific antibody. The arrow on the right indicates Sic1. (B) Degradation of Cdc6–LacZ versus Gcn4–LacZ. Cells carrying plasmid KB448

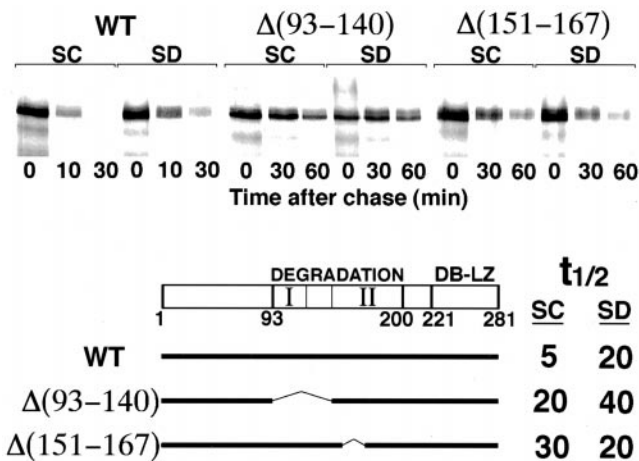


Figure 6. Effect of deletions in Gcn4 on the sensitivity to starvation. Full-length Gcn4 and two deletions fused to LacZ were subjected to pulse-chase analysis without (SC) or with (SD) prior mild starvation (15 min in medium lacking amino acids). Note the difference in time scale. $t_{1/2}$ indicates the half-lives of the three proteins under both conditions as derived from quantitation of the data by phosphorimager. "DEGRADATION" denotes the minimal region of Gcn4 required for degradation, and "I" and "II" indicate the two domains of that region, as defined by deletion analysis (see text). "DB-LZ" denotes the DNA binding domain and the leucine zipper domain of Gcn4.

Within domain I, point mutations at positions S101, T105, and P106 were sufficient to partially stabilize the protein (Kornitzer *et al.*, 1994). If this degradation signal were the only target modified in starved versus unstarved cells, then mutations in that signal would render the degradation unresponsive to starvation. However, we found that mutation T105A was still stabilized further by starvation (our unpublished results). Likewise, a protein lacking residues 93-140 was partially stabilized when compared with the full-length protein but still stabilized further under starvation conditions (Figure 6). In contrast, a deletion of residues 151-167 led also to partial stabilization of the protein in rich medium, but it was not stabilized further upon starvation; in fact, its half-life was somewhat reduced under starvation conditions (Figure 6). Degradation of $\Delta(151-167)$ was tested in two additional experiments and consistently found to be slightly faster under starvation conditions (unpublished results). Thus, deletion $\Delta(151-167)$ may define a sequence that is differentially modified in sated versus starved cells.

Gcn4 Degradation Requires Pho85 Activity

It was reasonable to assume that Gcn4 might be modified by phosphorylation because other known SCF substrates require phosphorylation by the main yeast cell cycle CDK, Cdc28, before ubiquitination. Although Gcn4 degradation is unaffected in *cdc28-1* mutant cells even at the nonpermissive temperature (Kornitzer and Fink, unpublished results), the yeast genome contains four additional CDK homologues, including Pho85, Kin28, and Srb10. An *SRB10* null mutant, *srb10-3* (Liao *et al.*, 1995), and a *KIN28* temperature-sensitive mutant, *kin28-ts3* (Valay *et al.*, 1993), were not defective in

Gcn4 degradation (unpublished results). We found, however, that *PHO85* is required for Gcn4 degradation. Initially, two observations suggested a genetic interaction between *PHO85* and *GCN4*. First, we found it impossible to transform a *pho85* Δ strain with a plasmid carrying a constitutive *GCN4* mutation (Hinnebusch, 1984). In addition, the *pho85* Δ mutant was highly sensitive to overexpression of *GCN4* under the *GAL* promoter (unpublished results).

These genetic interactions led us to test Gcn4 degradation in a *pho85* Δ mutant. As shown in Figure 7A, Gcn4 was markedly, but not completely, stabilized in the *pho85* Δ strain, with a half-life of 10 min instead of 2.5 min. In this assay, we used a Gcn4-LacZ fusion overexpressed under a heterologous promoter. To confirm that this stabilization was not due to overexpression, we used antibodies against Gcn4 to follow the degradation of the native, endogenous protein. Although the expression of the endogenous protein is weak, we were able to see a rapid degradation in the *PHO85* strain, and a strong stabilization in the *pho85* Δ strain (Figure 7C). We also tested transcriptional activity of endogenous Gcn4 in the *pho85* Δ mutant, using as reporter an *HIS4*-LacZ fusion exclusively dependent on Gcn4 for expression (Nagawa and Fink, 1985). *HIS4* expression was increased twofold in the mutant versus the wild-type strain, indicating that the stabilization of Gcn4 was accompanied by an increase in transcriptional activity (Figure 7D).

Identification of the Critical Pho85 Target Site on Gcn4

CDK target sites are characterized by a serine or threonine residue followed by a proline. Gcn4 contains five such sites, at positions 17, 61, 105, 165, and 218. Previous deletion analysis excluded positions 17, 61, and 218 from being required for Gcn4 degradation. Of the two remaining sites, Thr105 had already been shown to be required for degradation (Kornitzer *et al.*, 1994). However, the T105A mutant was still stabilized further in *PHO85* versus *pho85* Δ cells (Figure 7E), suggesting that Pho85 may target another site on Gcn4. The last potential site, Thr165, is located in domain II, shown by deletion analysis to be required for degradation, and for the starvation response (see above, Figure 6). Strikingly, Thr165 is embedded within a sequence, TPVL, which conforms to a proposed Pho85 target consensus sequence, S/TPXI/L (O'Neill *et al.*, 1996). To test the requirement of Thr165 for Gcn4 degradation, the residue was subjected to site-directed mutagenesis. Substitution of an alanine for Thr165 resulted in strong stabilization of the protein (Figure 8A), with an increase in half-life from 5 to >30 min. In addition, this mutant displayed a significant increase in Gcn4-dependent transcriptional activity, as measured by *HIS4*-LacZ activity (Figure 8B). The high initial stability of this mutant precluded us from testing for further stabilization upon starvation.

Phosphorylation of Thr165 in Vivo

To directly show Pho85-dependent phosphorylation of Thr165 in vivo, we fused a fragment of Gcn4 extending from residue 62 to residue 202 to a triple Myc epitope. This fragment, although more stable than full-length Gcn4, is still rapidly degraded (our unpublished results) and contains fewer potential kinase target sites than the full-length pro-

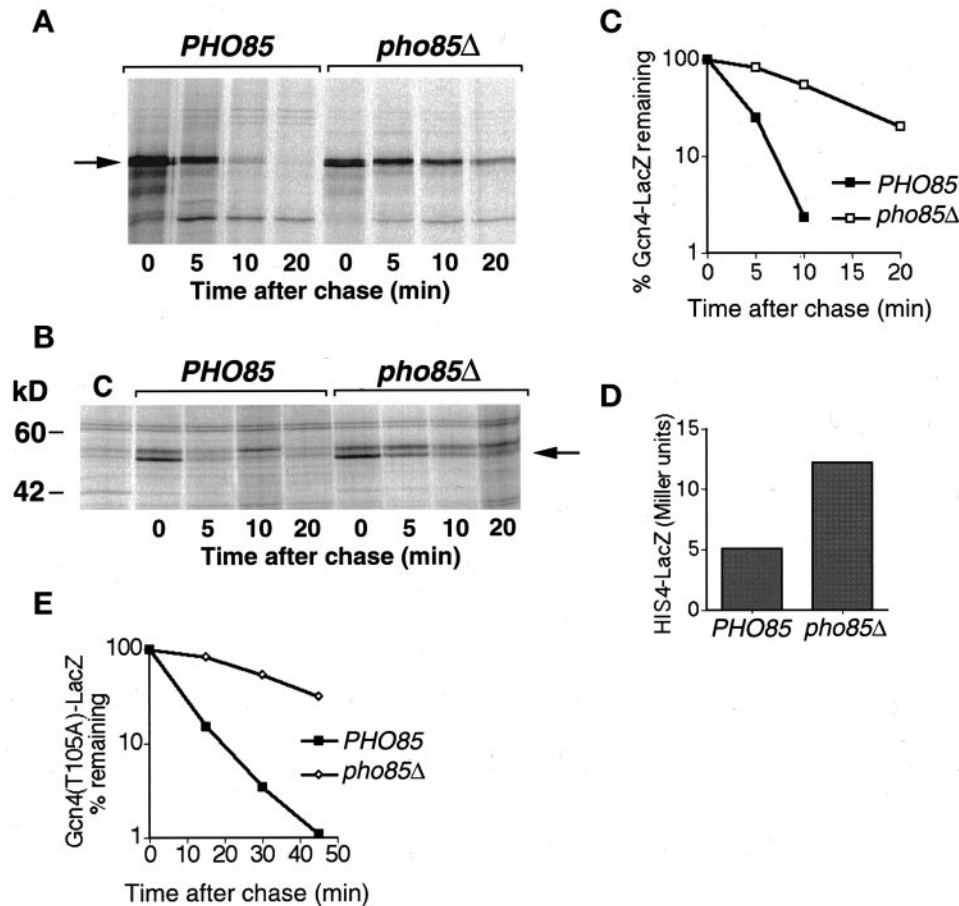


Figure 7. Degradation of Gcn4 in *pho85Δ* cells. (A) Pulse-chase analysis of Gcn4-LacZ expressed from the *CUP1* promoter of plasmid KB449 in W303 and DY4535 cells. (B) Quantitation of the experiment shown in (A). (C) Pulse-chase analysis of native, endogenous Gcn4 in W303 and DY4535 cells. The lane marked "C" indicates an extract from a *gcn4Δ* strain. The anomalous migration of native Gcn4 (shown here) as well as of recombinant Gcn4 (Figures 10 and 11), a 33-kDa protein, has been noted before (Hope and Struhl, 1985). Note that a doublet of cross-reacting bands migrates slightly slower than Gcn4. (D) Expression of His4-LacZ from a *HIS4* promoter derivative that is exclusively dependent on Gcn4 for activity (deletion number 203; Nagawa and Fink, 1985) in W303 and DY4535 cells. Overnight cultures were diluted and grown for 6 h to early log phase, and the β -galactosidase activity of the cultures was measured as described (Daignan-Fornier and Fink, 1992). (E) Degradation of the T105A mutant of Gcn4 in W303 and DY4535 cells. Gcn4(T105A)-LacZ was expressed from the GAL promoter of plasmid KB358 (Kornitzer *et al.*, 1994).

tein. The epitope-tagged fragment was then expressed in cells subjected to phosphate labeling and immunoprecipitated. As shown in Figure 9, (lanes 1 and 5) the immunoprecipitated phosphate-labeled protein migrated as two distinct bands, indicating that it still contained at least two phosphorylation sites. However, the protein fragment carrying the T165A mutation migrated as the single faster-migrating band only (lane 2), indicating that the slower-migrating band represents phosphorylation at Thr165 (the stronger signal of the mutant versus the wild-type protein fragment reflects the fact that the mutant protein is more stable). We then tested the phosphorylation pattern of Gcn4 in *pho85Δ* cells. Strikingly, the slower-migrating band was absent in these cells (lane 3), indicating that one site, presumably Thr165, is underphosphorylated in the *pho85Δ* mutant. We further tested phosphorylation of the 62–202 fragment under conditions that stabilize Gcn4. As shown in lanes 6 and 7, cycloheximide treatment or amino acid starvation also resulted in the disappearance of the slower-migrating band, suggesting loss of phosphorylation at Thr165 under these conditions.

Pho85 Phosphorylates Gcn4 in Vitro

The data shown above indicate that Gcn4 phosphorylation on Thr165 depends on Pho85 activity in vivo. However, the

possibility still existed that this is an indirect effect and that another kinase is directly responsible for Gcn4 phosphorylation. To show that Pho85 is able to directly phosphorylate Gcn4, we first attempted to reconstitute the phosphorylation reaction in vitro with pure recombinant proteins. Pho85, like all CDKs, requires a regulatory cyclin subunit for its activity. Pcl1 is one of the 10 different Pho85 cyclins (Pcls) identified to date (Andrews and Measday, 1998). We used recombinant GST-Pho85 together with GST-Pcl1 (Nishizawa *et al.*, 1998) to phosphorylate either full-length heptahistidine-tagged Gcn4 or the same 62–202 fragment of Gcn4 used for the in vivo phosphate labeling, fused to GST. As shown in Figure 10, efficient phosphorylation of full-length Gcn4 depended both on an active Pho85 kinase and on Pcl1. Furthermore, phosphorylation of the wild-type 62–202 Gcn4 fragment fused to GST yielded two bands, but with the mutant T165A construct only the lower band was evident, indicating that Pho85 is able to phosphorylate Thr165 directly.

Finally, we asked whether cellular Pho85 and Pcl1 activity toward Gcn4 was dependent on physiological conditions favoring Gcn4 degradation. ha-tagged Pcl1 (Nishizawa *et al.*, 1998) was immunoprecipitated from untreated exponentially growing cells, or from amino acid-starved or cycloheximide-treated cells. As shown in Figure 11, kinase activ-

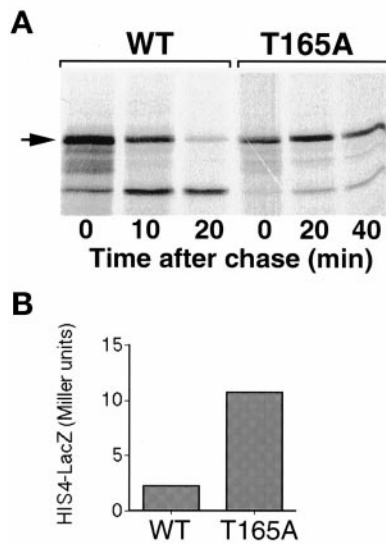


Figure 8. A mutation at residue Thr165 stabilizes Gcn4. (A) Pulse-chase analysis of wild-type (KB149) and mutant (KB854) Gcn4-LacZ expressed from the *GAL* promoter in W303 cells. Note the difference in time scale. (B) Expression of His4-LacZ from the full-length *HIS4* promoter (plasmid pFN6; Nagawa and Fink, 1985). The strain used, *bas1-2 bas2-2 gcn4Δ1* (KY26), is defective in both *HIS4* transcription factors (Arndt *et al.*, 1987). His4-LacZ expression is exclusively dependent on the plasmid-borne *GCN4* alleles expressed from the *ADE8* promoter of plasmids KB105 (WT) or KB853 (T165A). The β -galactosidase assay was performed as for Figure 7D.

ity toward both the full-length Gcn4 protein and the 62–202 fragment, was strongly reduced in the starved or cycloheximide-treated cells. Thus, Pcl1-associated Gcn4 kinase activity correlated with the conditions under which Gcn4 is rapidly degraded.

DISCUSSION

The findings presented here begin to unravel the mechanism by which Gcn4 degradation is regulated: a) inhibition of protein synthesis, even in the absence of starvation, is sufficient to stabilize Gcn4; b) the SCF^{CDC4} ubiquitination complex is required for Gcn4 degradation, but its activity is not affected by starvation; c) Gcn4 degradation requires the activity of the cyclin-dependent kinase Pho85; d) a specific domain of Gcn4 is required for its starvation-sensitive degradation; e) a specific site within that region, Thr165, is required for Gcn4 degradation; f) phosphorylation of this site depends on Pho85 activity and correlates with the conditions that allow rapid degradation of Gcn4; g) Pho85 is able to phosphorylate Thr165; and h) Pho85 activity toward Gcn4 is reduced in starved cells. The simple model emerging from these results is that Gcn4 phosphorylation by Pho85 on Thr165 transforms it into a substrate of the SCF^{CDC4} ubiquitination complex (Figure 12). The differential degradation of Gcn4 versus other SCF^{CDC4} substrates is explained by the different kinases required to transform the proteins into effective substrates of the ubiquitination complex. The regulation of Pho85 activity by starvation explains the stabilization of Gcn4 under these conditions.

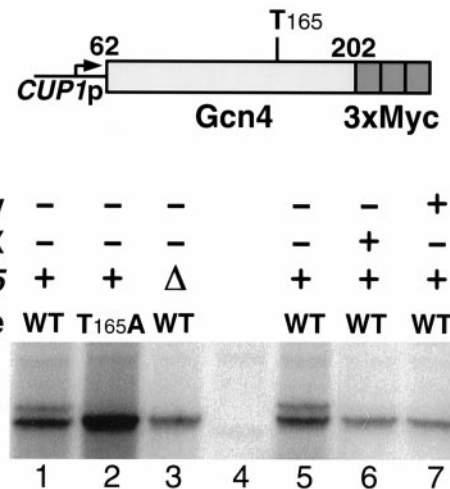


Figure 9. In vivo phosphorylation of a fragment of Gcn4 encompassing residues 62–202 fused to a triple Myc epitope and expressed from the *CUP1* promoter. The upper phosphorylation band (indicated by an arrow) is 30–40% as intense as the lower band in lanes 1 and 5 and is undetectable in the other lanes, even upon prolonged exposure (we estimate the limit of detection at <2% of the main band). Lane 4 is a no-tag control. CHX, cycloheximide treatment (1 μ g/ml, 30 min); a.a. starv, 30-min incubation in YNB medium lacking all amino acids.

Recognition sites of the SCF complexes are ill defined, beyond the general requirement for phosphorylation. Deletion analysis allowed to define two separate domains, I and II, within the region of Gcn4 required for its rapid degradation (Figure 7). We previously described a number of mutations in and around position 105 that stabilize Gcn4 (Kornitzer *et al.*, 1994). Thr105 is located in domain I. Thr165, which we characterize in the present study, is located in domain II. It is possible that these two residues constitute each the core of partially redundant SCF^{CDC4} recognition sites. Phosphorylation of the 62–202 fragment of Gcn4 (Figure 9), which carries both sites, suggests that in vivo, Pho85 activity is required for Thr165 phosphorylation but that phosphorylation of (an)other site(s) in this fragment, possibly Thr105, does not require Pho85. Conversely, our in vitro phosphorylation data (Figures 10 and 11) indicate that this same fragment can be phosphorylated by Pho85 at Thr165 as well as at another site, possibly Thr105. Thus, Pho85 activity is necessary and sufficient only for phosphorylation of Thr165.

Interestingly, contrary to Nishizawa *et al.* (1998), who found that recombinant Pho85-Pcl1 will phosphorylate Sic1 only after being “activated” with a yeast cell extract, we found that phosphorylation of Gcn4 does not require such an activation. Our results are in agreement with the recent findings of Wilson *et al.* (1999), that Gsy2 is efficiently phosphorylated in vitro by recombinant Pho85-Pcl10. Thus, it is possible that in the case of Sic1 phosphorylation, the cell extract confers a specificity factor required for Sic1 recognition, rather than an activation factor.

The Thr165 mutation was not isolated in our previous screen, probably because that screen depended on the enhanced transcriptional activity of Gcn4—the Thr105 mutation yielded a 15-fold increase in transcriptional activity

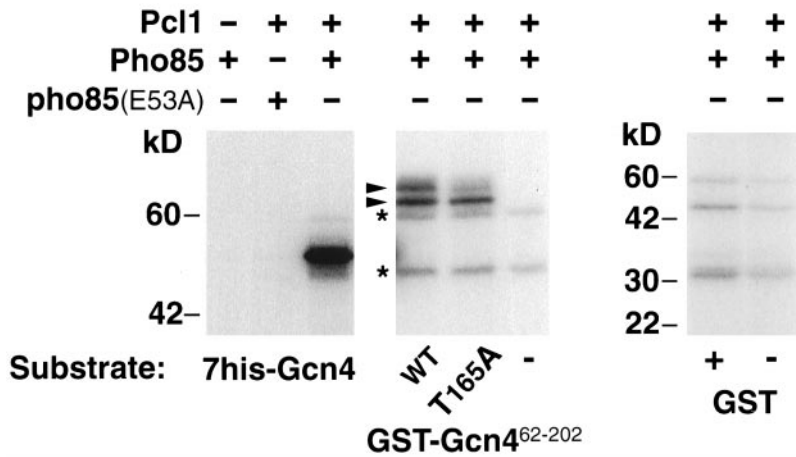


Figure 10. In vitro phosphorylation of Gcn4 by Pho85-Pcl1. Different substrates (indicated at the bottom) were incubated with wild-type or catalytically inactive (E53A) GST-Pho85, with or without GST-Pcl1. Asterisk indicates background phosphorylation on bacterial proteins copurifying with the recombinant kinase and cyclin. The two arrowheads indicate the two phosphorylation bands of GST-Gcn4⁶²⁻²⁰². The two weak signals (<10% intensity) visible over the main band in the T165A mutant presumably represent phosphorylation at alternative sites. The right panel shows that GST alone is not phosphorylated by Pho85-Pcl1 (the GST protein band would be expected to migrate slightly faster than the 30-kDa marker).

(Kornitzer *et al.*, 1994), whereas the Thr165 mutation only increased transcription 5-fold (Figure 8). Interestingly, Thr105 lies within the region defined by deletion analysis as the Gcn4 activation domain (Hope and Struhl, 1986; Hope *et al.*, 1988). More recent mutational mapping showed that the activation domain can be subdivided into two redundant subdomains, the N-terminal and central acidic activation domains (Drysdale *et al.*, 1995; Jackson *et al.*, 1996). The Thr105 region lies between these two subdomains. Thus, it is possible that the mutations that were isolated in that region, in addition to stabilizing the protein, increase its specific transcriptional activity by modification of the activation region. In line with the recent findings of Komeili and O’Shea (1999), showing that phosphorylation of the transcription factor Pho4 by Pho85 affects its transcriptional activity, it is conceivable that the role of phosphorylation at Thr105 is to modulate the transcriptional activity of Gcn4 more than its stability.

The main difference between the requirements of Gcn4 degradation compared with that of the cell cycle substrates of SCF is the specific kinase involved. Gcn4 degradation requires Pho85 activity, whereas the other known SCF substrates require Cdc28 activity. The known functions of Pho85 in association with specific cyclins relate to metabolic regulation, e.g., phosphate assimilation (cyclin: Pho80; Kaffman *et al.*, 1994), or glycogen synthesis (cyclins: Pcl8 and -10;

Huang *et al.*, 1998). In this respect, a function in Gcn4 degradation and therefore, indirectly, in amino acid metabolism, fits this pattern. The fact that the degradation of the cell cycle substrates of the SCF^{CDC4} complex depends on the phase of the cell cycle, whereas Gcn4 degradation is constitutive during the cell cycle, can be accounted for by difference in the kinase involved. Indeed, the nine Cdc28-associated cyclins, which confer its substrate specificity, are each present only during a limited window of each cell cycle. In contrast, most, but not all, of the 10 Pho85 cyclins are present throughout the cell cycle (Andrews and Measday, 1998). Strikingly however, Pcl1 is one of the Pho85 cyclins that is strongly cell cycle regulated, at least at the level of transcription (Measday *et al.*, 1997). This apparent contradiction would be resolved if different Pcls were able to promote Gcn4 phosphorylation. Two observations indicate that this is in fact the case: a) if Pcl1 were the only Pcl able to promote Gcn4 phosphorylation, then Gcn4 should be stabilized in a *pcl1Δ* mutant. However, a number of single and multiple combinations of *pcl* mutants that we tested, including *pcl1Δ*, are unaffected in Gcn4 degradation (our unpublished results); and b) preliminary data indicate that at least one other Pcl, Pho80, is able to promote Gcn4 phosphorylation on Thr165 by Pho85 in vitro (our unpublished results). These two findings indicate that there may be a high redundancy in the Pcls able to activate Pho85 for Gcn4 phosphorylation.

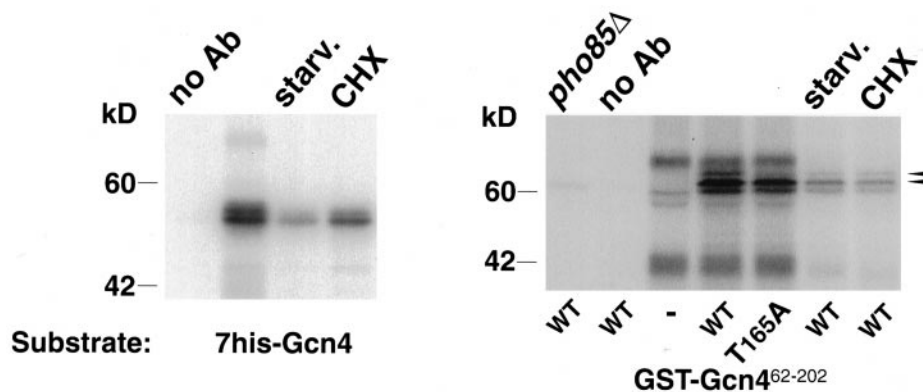


Figure 11. In vitro phosphorylation of Gcn4 by ha-Pcl1-associated kinase activity. Different substrates (indicated at the bottom) were incubated with Protein A-agarose beads carrying Pcl1-associated kinase activity immunoprecipitated from various cell extracts. *pho85Δ* indicates that the extract was made from a *pho85Δ* mutant strain; all other extracts were from wild-type cells. starv. and CHX refer to the same treatments as in Figure 9. no Ab refers to a blank immunoprecipitation in the absence of antibody. The two arrowheads at the right indicate the two phosphorylation bands of the GST-Gcn4⁶²⁻²⁰² substrate.

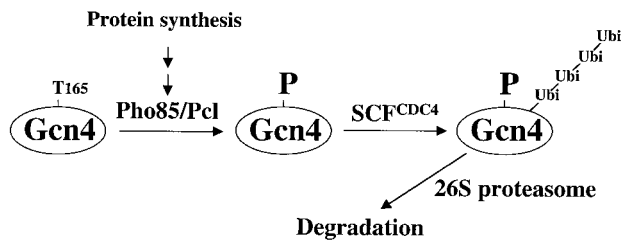


Figure 12. A model of the proposed pathway of regulation of Gcn4 degradation by starvation. See text for details.

How is Gcn4 phosphorylation by Pho85 regulated by starvation? Starvation could induce an inhibitor of Pho85 activity. Previous studies demonstrated that inhibition of an amino acyl-tRNA-charging enzyme also leads to Gcn4 stabilization (Kornitzer *et al.*, 1994), suggesting that uncharged tRNA constitutes the signal for Gcn4 stabilization. An elevated concentration of uncharged tRNA is also thought to constitute the primary signal for the translational control of Gcn4 (Wek *et al.*, 1995). In the current study, we found that inhibition of translation by cycloheximide is sufficient to stabilize Gcn4. Under these conditions, *charged* rather than uncharged tRNA accumulates in the cell. It should be noted that this does not contradict the earlier results obtained with the aminoacyl-tRNA-charging enzyme mutant; indeed, inhibition of one of the 20 aminoacyl tRNA synthetases, although resulting in an increase in the concentration of the uncharged form of its cognate tRNAs, would also, via inhibition of translation, increase the concentration of the *charged* form of all the other tRNAs. However, there is no direct evidence that Pho85 activity is regulated by the charged tRNA concentration or by any other signal generated by the stalled cellular biosynthetic machinery. An alternative possibility is that continued protein synthesis per se is required for maintaining Pho85 activity; therefore, starvation, or direct inhibition of protein synthesis, would result in reduced kinase activity.

Limitation of protein synthesis is known to arrest the cell cycle in G1 (Unger and Hartwell, 1976; Shilo *et al.*, 1978; Pardee, 1989). The fact that the SCF^{CDC4} complex is required for the G1/S transition and for Gcn4 turnover raised the possibility that amino acid starvation coordinately inhibits cell cycle progression and Gcn4 degradation via inhibition of the SCF^{CDC4} complex (Kornitzer *et al.*, 1994). However, our data do not support this hypothesis. Cdc6 and Sic1, two other substrates of the SCF^{CDC4} complex, are not stabilized upon starvation. Rather, our data indicate that Gcn4 degradation is regulated via modulation of Pho85 activity. Might modulation of Pho85 activity coordinately regulate Gcn4 turnover and cell cycle progression? Recent reports indicate a role for the G1 cyclin Cln3 in the cell cycle arrest in face of reduced protein synthesis (Polymenis and Schmidt, 1997; Danaie *et al.*, 1999). However, it cannot be excluded that modulation of Pho85 activity also participates in this regulation. Pho85, although not essential for cell cycle progression, displays cell cycle-related phenotypes. For example, a deletion of the Cdc28 cyclins *CLN1* and *CLN2* is synthetically lethal with a deletion of the Pho85 cyclins *PCL1* and *PCL2* or of *PHO85* itself (Espinoza *et al.*, 1994; Measday *et al.*, 1994). Thus, it is possible that Pho85 is one of the transducers

of physiological signals, such as amino acid starvation, that need to be integrated by the cell cycle machinery.

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