Direct Inhibition of the Yeast Cyclin-Dependent Kinase Cdc28-Cln by Far1

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Cell cycle arrest of *Saccharomyces cerevisiae* in G_1 by the antimitogen α-factor is mediated by activation of a signal transduction pathway that results in inhibition of the cyclin-dependent kinase Cdc28-Cln. The Far1 protein is required for cell cycle arrest and associates with the Cdc28-Cln complex. The kinase activity of Cdc28-Cln was directly inhibited by Far1 both in vivo and in vitro, thus demonstrating that Far1 acts at the final step in the α-factor response pathway by inhibiting a G_1 cyclin-dependent kinase.

Each haploid cell type of the yeast *S. cerevisiae* secretes a specific peptide pheromone (**a**- or α-factor) that activates a signal transduction pathway and thereby induces changes in gene transcription, alterations of cellular morphology, and cell cycle arrest in G_1 (*1*). This pathway is initiated by binding of the pheromone to a receptor coupled to a heterotrimeric guanosine triphosphate–binding protein (G protein). The signal is subsequently propagated by an unknown mechanism to a cascade of protein kinases (*2*). The pathway terminates with Ste12, a transcription factor that induces the expression of several genes by binding to their upstream regulatory regions (*3*).

Cell cycle arrest in response to α-factor results from inactivation of the Cdc28 protein kinase, which is necessary for progression from G_1 into S phase (*4*). The Far1 protein is specifically required for pheromone-induced cell cycle arrest (*5*). Far1 directly links the pheromone-triggered signal transduction pathway to the cell cycle machinery by associating with the protein kinase Cdc28-Cln in an α-factor–dependent manner (*6*). The biological relevance of this association is indicated by the correlation between the abilities of *far1* mutants to form the complex and to induce cell cycle arrest in vivo.

There are several requirements for activity of the Cdc28-Cln protein kinase, any of which could be the target for inhibition by Far1. Active kinase requires association of the catalytic subunit, Cdc28, with a G_1 cyclin encoded by one of the *CLN* genes (*7*). Furthermore, the Cdc28 subunit must be phosphorylated on Thr^{169} and unphosphorylated on Tyr^{19} (*8*). Inhibition of kinase activity in response to α-factor could therefore occur by degradation or dissociation of the cyclin subunit, or by changing the phosphorylation state of Cdc28. Activity of the Cdc28-containing kinase could also be inhibited by binding of an inhibitory protein such as p40 (*9*).

To determine whether Far1 acts by promoting degradation of the G_1 cyclins, we expressed the *CLN2* gene constitutively from the *ADH1* regulatory region, so that transcription of *CLN2* was uncoupled from control by Cdc28 kinase (*10*). Cells constitutively expressing an epitope-tagged version of Cln2 (Cln2-HA) (*11*) exhibited cell cycle arrest in response to α-factor with kinetics indistinguishable from those of control cells (Fig. 1A) (*12*). These cells contained similar amounts of Cln2 protein (*13*), which decayed with comparable half-lives (~5 min) (*14*) whether or not the cells were arrested with α-factor (Fig. 1B). In contrast, when *CLN2* expression was under the control of its own promoter, Cln2 protein rapidly disappeared after addition of α-factor in a *FAR1*-dependent manner (Fig. 1C) (*12*). These results demonstrate that α-factor is able to induce cell cycle arrest in cells containing substantial amounts of Cln2 protein and indicate that Far1 does not trigger degradation of Cln2.

To examine the possibility that Far1 affects the association of Cln2 and Cdc28 in vivo, we immunoprecipitated Cln2 from arrested or growing cells and analyzed the immunoprecipitates for the presence of Cdc28 protein by immunoblotting (*13*). Similar amounts of Cdc28 were co-immunoprecipitated with Cln2 whether or not the cells were arrested with α-factor (Fig. 2A). Most of the Cln2 protein migrated as a multiply phosphorylated species in both arrested and growing cells (Fig. 1, B and C; Fig. 2B) (*15*), as expected for Cln2 complexed with Cdc28 (*14*, *16*). These observations indicate that α-factor does not influence complex formation between Cln2 and Cdc28 and thus that Far1 does not interfere with their association.

Cyclin-dependent kinases (CDKs) are regulated both positively and negatively by phosphorylation (*8*). Phosphorylation of Tyr^{19} and Thr^{18} in the adenosine triphosphate (ATP)–binding domain of Cdc28 inhibits its kinase activity (*17*). However, Cdc28 is not phosphorylated on these residues in α-factor–arrested cells (*18*). Furthermore, cells expressing a mutant form of Cdc28 that cannot be phosphorylated on these residues are still arrested normally in response to α-factor (*14*, *18*). Thus, Far1 does not appear to promote inhibitory phosphorylation of these Cdc28 residues.

Phosphorylation of Cdc28 on Thr^{169} is mediated by CAK (CDK activating kinase), and phosphorylation of this residue is required for kinase activity (*19*). Therefore, Far1 could interfere with phosphorylation of Thr^{169} or be a direct inhibitor of the kinase activity of Cdc28-Cln. If α-factor reduced Cdc28-Cln activity by blocking phosphorylation of Thr^{169}, Cln-associated kinase activity would be low irrespective of the conditions used to isolate the kinase. If, on the other hand, Cdc28-Cln were inactivated by an associated inhibitor, we might be able to dissociate this inhibitor during isolation of the Cln-associated kinase and recover fully active kinase from arrested cells. Therefore, Cdc28-Cln2 complexes were immunoprecipitated from extracts prepared from untreated cells or from cells

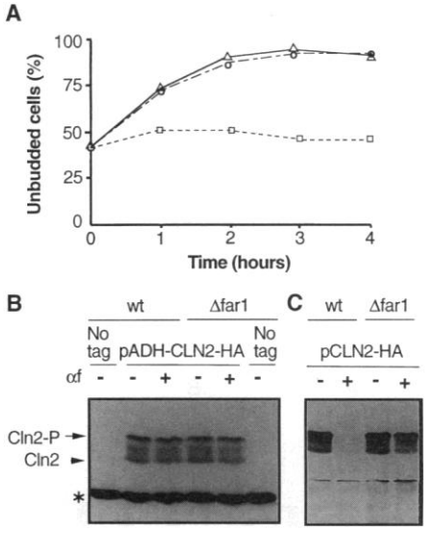

Fig. 1. Stability of Cln2 in cells exposed to α-factor. (**A**) Wild-type cells (○) or a *far1* deletion strain (□) harboring a plasmid encoding Cln2-HA under the control of the constitutive *ADH1* promoter or wild-type cells expressing a control plasmid (△) were treated with α-factor (1 μg/ml). The percentage of unbudded cells was determined microscopically at the indicated times. (**B** and **C**) Amounts of Cln2 protein in crude extracts prepared from wild-type (wt) or *far1* deletion (Δfar1) strains expressing Cln2 with (pADH-CLN2-HA or pCLN2-HA) or without (No tag) the HA epitope were analyzed by immunoblotting with 12CA5 antibodies. Cells were treated with (+) or without (−) α-factor (1 μg/ml) (αf) for 3 hours; arrest was confirmed microscopically. Arrowhead, unphosphorylated Cln2-HA (Cln2); arrow, fully phosphorylated Cln2 (Cln2-P); asterisk, a protein that cross-reacts with the 12CA5 antibody. Cln2 was expressed from the constitutive *ADH1* promoter (B) or from its own promoter (C).

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arrested with α-factor and were washed either gently or under stringent conditions (*20*). The kinase activities of the Cln2 immunoprecipitates were then assayed with histone H1 as a substrate. After mild washing, Cln2 immunoprecipitates from α-factor–arrested cells showed one-fifth to one-third the amount of histone H1 kinase activity as immunoprecipitates from exponentially growing cells (Fig. 2, D and E). This decrease in activity was dependent on Far1; no difference in histone H1 kinase activity was detected for cells lacking Far1. In contrast, after stringent washing, immunoprecipitates from both wild-type and *far1* mutant cells showed similar histone H1 kinase activities irrespective of whether the cells were arrested with α-factor or not (Fig. 2C). The stringent washing conditions removed associated Far1 from the Cdc28-Cln2 complex (Fig. 2F). Thus, the Cdc28-Cln2 complex in α-factor–arrested cells is present in a potentially active form and therefore must be phosphorylated on Thr^{169}. These observations are consistent with the idea that cell cycle arrest by α-factor is accompanied by a decrease in Cdc28-Cln2 activity due to an associated inhibitor.

To test directly whether association of Far1 with the Cdc28-Cln2 complex inhibited kinase activity, we purified Far1 from yeast cells induced for the pheromone pathway (*21*) and determined its ability to inhibit Cdc28-Cln2 kinase activity in vitro (*22*). Far1 was tagged at its COOH-terminus with the 6His element to facilitate purification and was expressed from the inducible *GAL1* promoter (Fig. 3A) (*21*). Fractions containing purified Far1 inhibited immunoprecipitated Cdc28-Cln2 protein kinase efficiently in vitro; no inhibition was detected with material from cells that did not produce Far1 (Fig. 3B). The kinase activity of the Cdc28-Cln2 complex exposed to Far1 was ~10% of that of the uninhibited complex (Fig. 3C).

To determine whether Far1 is sufficient to inhibit the kinase activity of Cdc28-Cln2 in vitro, we expressed various versions of Far1 as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* (*23*). The wild-type GST-Far1 fusion protein used here is fully active and complements a *far1* deletion mutant when expressed in yeast (*6*). Prior studies have shown that high concentrations of Far1 protein are not sufficient to induce cell cycle arrest (*24*); phosphorylation of Far1 is thought to be necessary for its activity in vivo (*6*). We therefore purified a mutant form of Far1 from *E. coli* that is constitutively active. Yeast cells that express this mutant protein, Far1-22, from the inducible *GAL1* promoter were arrested in the absence of α-factor (Fig. 4A). In contrast, cells producing either wild-type Far1 or a mutant Far1 protein (Far1-60F3) that binds only weakly to the Cdc28-Cln2 complex grew normally (Fig. 4A) (*6*). Purified GST–Far1-22 protein isolated from *E. coli* inhibited the kinase activity of Cdc28-Cln2 and Cdc28-Cln1 in vitro in a dose-dependent manner (Fig. 4, B and C; Fig. 5) (*22*); GST alone had no effect. Purified GST–Far1-60F3 inhibited the activity of Cdc28-Cln2 only at high concentrations (Fig. 4C), suggesting that inhibition of Cdc28-Cln kinase requires binding of Far1 to the kinase. GST–Far1-22 did not inhibit the activity of the mitotic Cdc28-Clb2 kinase or the S phase Cdc28-Clb5 kinase (Fig. 5). Together, these results demonstrate that Far1 is a direct and specific inhibitor of the Cdc28-Cln protein kinase in vitro.

Down-regulation of the kinase activity of Cdc28-Cln in response to α-factor in vivo is achieved both by direct inhibition by Far1 and by a decrease in the amount of *CLN1* and *CLN2* transcripts, which results in a loss of Cln1 and Cln2 proteins (*7*, *10*). This second mode of inhibition may also result from the action of Far1. Because transcription of *CLN1* and *CLN2* is controlled by a positive feedback loop driven by Cdc28-Cln protein kinase (*10*), inhibition of kinase activity by Far1 may interrupt this loop and thereby reduce expression of Cln proteins. A decreased amount of Cln pro-

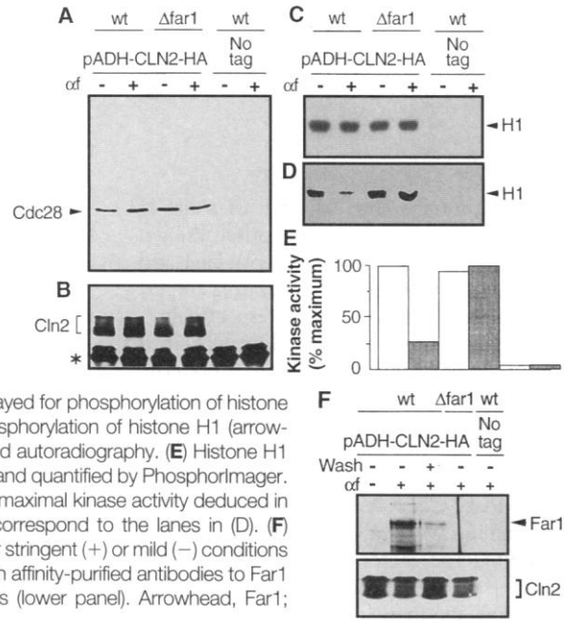

Fig. 2. Effect of α-factor on association of Cdc28 with Cln2 and on association of an inhibitory factor with Cdc28-Cln2. Epitope-tagged Cln2 was immunoprecipitated with 12CA5 antibodies from extracts prepared from wild-type (wt) or *far1* deletion (Δfar1) strains either arrested (+) or not arrested (−) with α-factor (αf). (**A** and **B**) Immunoprecipitates were immunoblotted with affinity-purified antibodies to Cdc28 (A) or with 12CA5 antibodies (B). Arrowhead, Cdc28; bracket, Cln2-HA (Cln2); asterisk, immunoglobulin G. (**C** and **D**) Immunoprecipitates were washed under stringent (C) or mild (D) conditions (*20*) and then assayed for phosphorylation of histone H1 in the presence of [γ-^{32}P]ATP. Phosphorylation of histone H1 (arrowhead) was monitored by SDS-PAGE and autoradiography. (**E**) Histone H1 kinase assays were performed as in (D) and quantified by PhosphorImager. Data are expressed as a percentage of maximal kinase activity deduced in three independent experiments; bars correspond to the lanes in (D). (**F**) Immunoprecipitates were washed under stringent (+) or mild (−) conditions as in (C) and (D) and immunoblotted with affinity-purified antibodies to Far1 (upper panel) or with 12CA5 antibodies (lower panel). Arrowhead, Far1; bracket, Cln2-HA (Cln2).

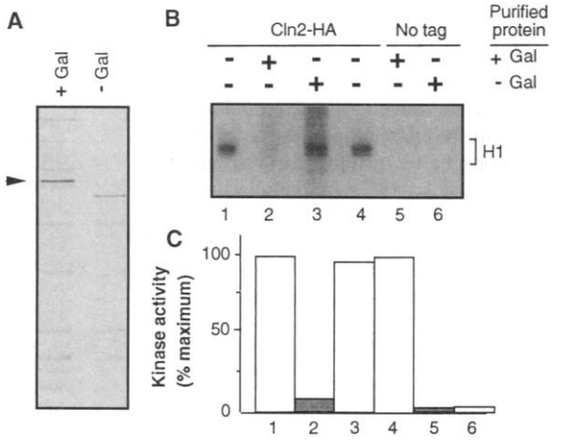

Fig. 3. Inhibition of Cdc28-Cln2 protein kinase in vitro by Far1 partially purified from yeast. (**A**) Far1 tagged with a 6His element was expressed in yeast from the inducible *GAL* promoter and purified from cells grown in the presence (+Gal) or absence (−Gal) of galactose. The strain also carries *GAL-STE4*, which allows induction of the pheromone response pathway by galactose (*28*). The final preparation (~60% pure) was subjected to SDS-PAGE and stained with silver. Arrowhead, Far1-6His. (**B**) Epitope-tagged Cln2 (Cln2-HA) was immunoprecipitated as described (*22*) and incubated either with fractions containing purified Far1 (lane 2), an equal volume of control preparation (lane 3), or buffer alone (lanes 1 and 4). Histone H1 kinase assays were performed in the presence of [γ-^{32}P]ATP and analyzed as described (*20*). Control immunoprecipitates from cells expressing Cln2 without the HA epitope were also assayed (lanes 5 and 6). (**C**) Three independent experiments were performed as described in (B). Phosphorylated histone H1 was excised from the SDS-polyacrylamide gels and incorporated radioactivity was determined (Cerenkov counts). Activity in buffer controls was set as 100%. Bar numbers correspond to lane numbers in (B).

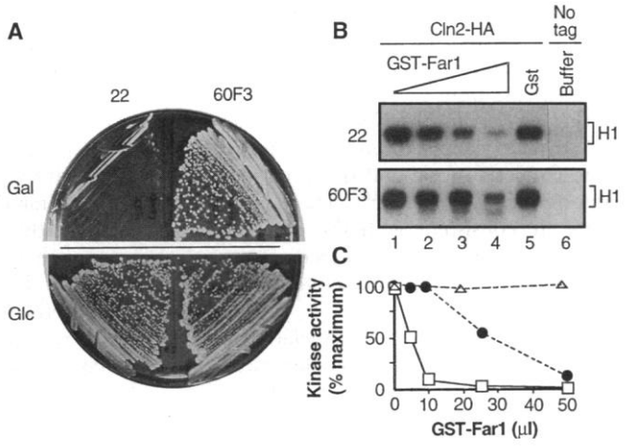

Fig. 4. Effects of an activated Far1 protein and a Far1 mutant on Cdc28-Cln2 protein kinase activity in vivo and in vitro. (**A**) A wild-type yeast strain, expressing from the inducible *GAL* promoter either a constitutively active Far1 protein (22) or a Far1 mutant (60F3) that binds only weakly to the Cdc28-Cln2 kinase, was grown on plates containing galactose (Gal, upper panel) or glucose (Glc, lower panel). (**B**) GST–Far1-22, GST–Far1-60F3, and GST proteins were purified from *E. coli* and incubated with immunoprecipitated Cdc28-Cln2. Histone H1 kinase activity was determined as described (*20*). Bracket, histone H1. (**C**) Two independent experiments were performed as in (B). Phosphorylated histone H1 was excised from gels and incorporated radioactivity was quantified by measuring Cerenkov counts. Maximal (100%) activity corresponds to counts per minute incorporated into histone H1 after addition of buffer to the Cdc28-Cln2 immunoprecipitates. Values are means of both experiments. □, GST–Far1-22; ●, GST–Far1-60F3; △, GST.

teins is apparently not absolutely required for cell cycle arrest in response to α-factor, because we have shown that Far1 can promote cell cycle arrest even when Cln2 is present in large amounts. Arrest under these conditions appears to be weaker than in the wild-type situation: Cells were able to form colonies after long incubation on plates containing α-factor (*14*). A decrease in Cln protein concentration in α-factor–treated cells thus appears to contribute to stable arrest.

Genetic and biochemical evidence suggests that Far1 inhibits Cdc28-Cln1 and Cdc28-Cln2 in vivo and vitro (Figs. 4 and 5) (*5*, *12*). In contrast, Far1 does not appear to affect Cdc28-Cln3; although Far1 associates with Cdc28-Cln3 after prolonged α-factor arrest, no change in kinase activity is apparent (*25*). Similarly, Far1 does not inhibit mitotic Cdc28 complexes containing Clb proteins (Fig. 5), at least some of which are inhibited by the other known Cdc28 inhibitory protein, p40 (*9*). Far1 and p40 may thus be specific inhibitors for G_1 and mitotic forms of Cdc28, respectively.

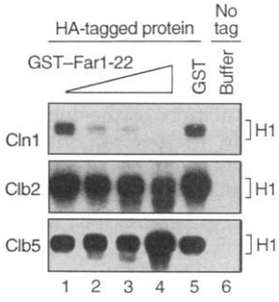

Fig. 5. Effects of an activated Far1 protein on the kinase activity of Cdc28 complexed with Cln1, Clb2, or Clb5. Epitope-tagged Cln1, Clb2, and Clb5 were immunoprecipitated as described (*22*) and incubated with purified GST–Far1-22 or GST as in Fig. 4B Histone H1 kinase activity was determined as described (*20*). Bracket, histone H1.

We now have substantial insight into the molecular mechanisms that underlie cell cycle arrest in response to an antimitogenic factor in yeast: The mating factor response pathway activates Far1, and Far1 then inhibits the essential cyclin-dependent kinase Cdc28-Cln. Cell cycle arrest of mammalian Mv1Lu epithelial cells by the antimitogenic factor transforming growth factor–β (TGF-β) may be mediated in an analogous manner. In this case, TGF-β induces inactivation of the cyclin-dependent kinase Cdk2–cyclin E as a result of the action of the inhibitory protein $p27^{kip1}$ (*26*). Several other inhibitors of CDKs have been identified, some of which are deficient in cancer cells (*27*). It appears, therefore, that in yeast, as well as in mammalian cells, CDKs are regulated by expression or activation of specific inhibitors in response to a variety of biological signals including antimitogenic factors and feedback control mechanisms.

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11. Cln2-HA consists of the Cln2 coding sequence followed by three copies of the hemagglutinin (HA) epitope tag [J. Field *et al.*, *Mol. Cell. Biol.* **8**, 2159 (1988)]. Expression of Cln2-HA was under the control of either the *ADH1* promoter (pTP45) or the *CLN2* promoter (pTP35) (*6*) on a pRS316 CEN/ARS plasmid [R. S. Sikorsky and P. Hieter, *Genetics* **122**, 19 (1989)]. Both plasmids fully complemented a strain deleted for *CLN1*, *CLN2*, and *CLN3* (*14*).
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13. Cell lysates were prepared and Cln2-HA was immunoprecipitated with 12CA5 antibodies (BAbCo) as described (*6*). Immunoprecipitated proteins were eluted with SDS sample buffer, boiled for 5 min, and resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel). Immunoblotting procedures were as described (*6*).
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20. Wild-type (FC279) or *far1* deletion (YMP18) strains harboring the plasmid pTP45 were grown in SD-URA medium [J. B. Hicks and I. Herskowitz, *Genetics* **83**, 245 (1976)] in the absence or presence of α-factor (1 μg/ml) for 3 hours. A portion of the cells was fixed and briefly sonicated, and the percentage of unbudded cells was determined microscopically. The remaining cells were lysed in ice-cold buffer N [50 mM tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.1% NP-40, 10 mM NaF, 60 mM β-glycerophosphate, 1% aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 1 mM leupeptin], and the lysate was subjected to immunoprecipitation with 12CA5 antibodies (*6*). Immunoprecipitates were washed twice with buffer N and divided; one-half was incubated for 30 min in buffer S (stringent) [50 mM tris-HCl (pH 7.5), 500 mM NaCl, 1% Triton X-100, 0.025% SDS, 10 mM NaF, 60 mM β-glycerophosphate, 1% aprotinin, 0.1 mM PMSF, 2 mM benzamidine, 1 mM leupeptin], the other half was incubated for 30 min in buffer M (mild) [50 mM tris-HCl (pH 7.5), 100 mM NaCl, 10 mM NaF, 60 mM β-glycerophosphate, 1% aprotinin, 0.1 mM PMSF, 2 mM benzamidine, 1 mM leupeptin]. Immunoprecipitates were then washed three times with the respective incubation buffers and twice with kinase buffer [10 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1% aprotinin, 0.1 mM PMSF, 2 mM benzamidine, 1 mM leupeptin]. Kinase reactions were started by addition of 50 μl of a prewarmed solution containing 10 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 μM ATP, histone

H1 (0.25 mg/ml) (Boehringer Mannheim), and 10 μCi of [γ-^{32}P]ATP (Amersham). After incubation for 10 min at 30°C, the reaction mixture was briefly centrifuged, and 50 μl of SDS sample buffer were added to the supernatant, which was then boiled for 5 min. Proteins were separated by SDS-PAGE (12% gel), and ^{32}P-labeled histone H1 was visualized by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

21. RD 680 (*MATα ura3-52 leu2-3,112::GAL-STE4::LEU2 reg1-501 GAL$^+$ pep4-3 prd1-1122*) transformed with pTP20 (p*GAL-FAR1*-6His; this plasmid fully complemented a *far1* deletion strain) was grown in SD-URA to mid–logarithmic phase before expression of Far1, and Ste4 was induced by adding galactose (2%). Exposure of these cells to galactose induces the mating pathway because of overexpression of Ste4 (*28*) and allows production of Far1 in its fully phosphorylated and presumably active form (*6, 23*). As a control, we performed the same purification protocol with extracts prepared from cells that were not exposed to galactose and therefore did not produce Far1. After incubation for 5 hours with (or without) galactose, cells were centrifuged and extracts were prepared as described [M. Woonter, P. A. Wade, J. Bonner, J. A. Jaehning, *Mol. Cell. Biol.* **11**, 4555 (1991)]. The $(NH_4)_2SO_4$ pellet was resuspended in 20 mM Hepes-KOH (pH 7.5), 150 mM potassium glutamate, 10 mM $MgSO_4$, 10 mM EGTA, 20% glycerol, 5 mM PMSF, 2 mM benzamidine, 80 mM β-glycerophosphate, 10 mM NaF, and 5 mM dithiothreitol (DTT) and was dialyzed overnight against the same buffer at 4°C; portions were then frozen. The dialyzed extract was diluted in HNG binding buffer [20 mM Hepes (pH 7.8), 500 mM NaCl, 0.5% NP-40, 10% glycerol, 10 mM imidazole, 10 mM NaF, 60 mM β-glycerophosphate, 1% aprotinin, 0.1 mM PMSF, 2 mM benzamidine, 1 mM leupeptin] and applied to a column containing iminodiacetic acid immobilized on Sepharose-CLB (Sigma) coupled with Ni^{2+}. The column was washed with HNG buffer, and proteins were eluted with 10 ml of 0.5× HNG containing 200 mM imidazole. The eluate was diluted with 30 ml of HG buffer [20 mM Hepes-KOH (pH 7.5), 10 mM $MgSO_4$, 10 mM EGTA, 20% glycerol, 5 mM PMSF, 2 mM benzamidine, 80 mM β-glycerophosphate, 10 mM NaF, 5 mM DTT] and applied to a HiTrap Q column (Pharmacia). The column was washed extensively with HG buffer containing 100 mM NaCl, and proteins were eluted with HG buffer containing 500 mM NaCl. The eluate was diluted with HG buffer and applied to a HiTrap SP column (Pharmacia). The column was washed with HG buffer containing 100 mM NaCl, and bound proteins were eluted with HG buffer containing 500 mM NaCl. Fractions containing Far1-6His were then dialyzed against 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 2 mM PMSF, and 2 mM benzamidine, concentrated in a Centricon 30 microconcentrator (Amicon), and frozen in portions at −70°C.
22. Cdc28-Cln2 was immunoprecipitated from extracts prepared from YMP29 (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL$^+$ psi$^+$ cln2::CLN2-HA-LEU2 far1::URA3*) as described (*6*). Immunoprecipitates were washed four times with buffer S (*20*) and twice with kinase buffer and divided into equal portions. One portion was tested for the presence of Cln2-HA by immunoblotting. Immunoprecipitates were incubated with purified Far1 for 30 min at 4°C in kinase buffer and then either washed gently with kinase buffer or used directly to assay H1 kinase activity as described (*20*). Phosphorylated histone H1 was excised from SDS-polyacrylamide gels and incorporated radioactivity was measured (Cerenkov counts). Full activity (100%) corresponds to counts per minute incorporated into histone H1 in reactions in which only buffer was added. Cdc28-Cln1, Cdc28-Clb2, and Cdc28-Clb5 complexes were immunoprecipitated with 12CA5 antibodies as described above from the following strains: YMT297 (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL$^+$ psi$^+$ cln1::CLN1-HA*) (kindly provided by M. Tyers, University of Toronto; YMG5 (*MAT***a** *ade2-1 trp1-1::GAL-CLB2-HA-TRP1 can1-100 leu2-3,112 his3-11,15 ura3 GAL$^+$ psi$^+$*) (kindly provided by M. Glotzer, EMBL, Heidelberg); and K3819 (*MAT***a** *ade2-11 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3::ADH-HA-CLB5-URA3 GAL$^+$ psi$^+$*) (kindly provided by E. Schwob, IMP, Vienna).
23. The coding sequences corresponding to the NH_2-terminal 390 amino acids of the *FAR1* alleles 60F3 and 22 (starting from the corrected *Far1* NH_2-terminus [F. Cross, personal communication]) were cloned into the *E. coli* expression vector pGEX1 (Pharmacia). The GST fusion proteins were expressed in *E. coli* NB42 and purified by affinity chromatography on gluthathione–Sepharose 4B (Pharmacia) as described (*6*). Eluted fractions were dialyzed against 20 mM tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 2 mM PMSF, and 2 mM benzamidine, concentrated in a Centricon 30 microconcentrator, and frozen in portions. Concentrations of the purified GST-Far1 proteins were equalized (to approximately 5 μg/ml) after immunoblotting with antibodies to GST. Samples were used in H1 kinase assays as described (*22*).
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cDNA Cloning and Interferon γ Down-Regulation of Proteasomal Subunits X and Y

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Proteasomes are the proteolytic complex responsible for major histocompatibility complex (MHC) class I–restricted antigen presentation. Interferon γ treatment increases expression of MHC-encoded LMP2 and LMP7 subunits of the proteasome and decreases expression of two proteasome subunits, named X and Y, which alters the proteolytic specificity of proteasomes. Molecular cloning of complementary DNAs encoding X and Y showed that their proteins are proteasomal subunits with high amino acid similarity to LMP7 and LMP2, respectively. Thus, interferon γ may induce subunit replacements of X and Y by LMP7 and LMP2, respectively, producing proteasomes perhaps more appropriate for the immunological processing of endogenous antigens.

Cytosolic proteasomes, which function as an extralysosomal, adenosine triphosphate–dependent system for selectively degrading ubiquitinated proteins, are proposed to be involved in the processing of intracellular antigens into short peptides, which are then transported into the endoplasmic reticulum through the TAP1/2 heterodimeric peptide transporter (*1*). Two proteasomal polymorphic genes, LMP2 and LMP7, are located in the MHC class II region, closely linked to the TAP1 and TAP2 genes, and are up-regulated by interferon γ (IFN-γ), a major immunomodulatory cytokine (*2*). Treatment with IFN-γ induces a change in specificity of proteasomes for peptide degradation that is possibly responsible for effective generation of antigenic peptides associated with cell surface class I molecules (*3, 4*), but it is unknown how the specificity of proteasomes is changed. Not only is the synthesis of LMP2 and LMP7 induced, but the synthesis of two proteasome subunits, tentatively named X and Y, is reduced (*4*). To clarify the role of IFN-γ, we isolated and characterized cDNAs encoding X and Y.

For identification of proteasome subunits X and Y, the exact subunit composition of the human 20S proteasome was examined by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The enzyme from human kidney was separated into multiple components of different sizes and charges (Fig. 1). Protein spots corresponding to LMP2 and LMP7, which were identified as newly synthesized subunits that were induced by IFN-γ treatment, were detected by immunoblot analysis with anti-peptide antibodies to LMP2 and LMP7 (*4*).

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