tions below 1 μM, we added 5 mM EGTA to the cytoplasmic solution and added $CaCl_2$ as calculated using the CaBuf program and published stability constants [A. Fabiato and F. Fabiato, *J. Physiol. (London)* **75**, 463 (1979)]. For experiments in which $Mg^{2+}$ was added to the cytoplasmic solution, $MgCl_2$ was added to the total concentrations stated in the text. Under these conditions, binding of $Mg^{2+}$ to gluconate is negligible (stability constant, 1.7 $M^{-1}$). Electrodes were pulled from thin-walled, filamented borosilicate glass (World Precision Instruments) and filled with 116 mM potassium gluconate, 4 mM KCl, 10 mM Hepes (pH 7.25). Electrode resistance was typically 2 to 5 megohms. Membrane patches were voltage-clamped with an Axopatch 200A amplifier (Axon Instruments). The data were low-pass–Bessel filtered at 2 kHz and were acquired with Pulse software (Heka Electronik). In the analysis we used Pulse, Kaleidograph (Abelbeck), or IGOR (Wavemetrics) software. All experiments were performed at room temperature from a holding potential of −80 mV. Voltage ramps (2.5 s) from −100 to 100 mV were acquired at a sampling frequency of 500 Hz. The currents recorded from macropatches showed small inward rectification in the absence of cytoplasmic cations other than $K^+$ and $Ca^{2+}$ (5 μM). In the hippocampus, SK channels exhibit significant inward rectification in the presence of intracellular $Mg^{2+}$ (*8*). When different concentrations of $Mg^{2+}$ (0.1 to 3 mM) were added to the intracellular face of inside-out patches, outward currents were reduced. Apamin was from Calbiochem, and *d*-tubocurare was from Research Biochemicals International.

28. Solutions used were the same as for macropatch recordings. Electrodes were pulled from Corning 7052 glass (Garner) and had resistances of 9 to 13 megohms. Data were filtered at 1 kHz (Bessel), acquired at 10 kHz with Pulse (Heka Electronik), and stored directly on a Macintosh Quadra 650. Single channels were analyzed with MacTac (SKALAR Instruments). The "50% threshold" technique was used to estimate event amplitudes. The threshold was adjusted for each opening, and each transition was inspected visually before being accepted. Amplitude histograms were constructed using MacTacfit (SKALAR Instruments), and the single channel conductance was determined from a Gaussian distribution. Channel open probability was estimated from *NP*(o), the product of the open probability multiplied by the number of channels; *NP*(o) was calculated as the sum of the (dwell time multiplied by level number) divided by the total time, and *N* was estimated as the number of simultaneously open channels at 0.4 μM $Ca^{2+}$.


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# A Cyclin-Dependent Kinase–Activating Kinase (CAK) in Budding Yeast Unrelated to Vertebrate CAK


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Progress through the cell cycle is governed by the cyclin-dependent kinases (CDKs), the activation of which requires phosphorylation by the CDK-activating kinase (CAK). In vertebrates, CAK is a trimeric enzyme containing CDK7, cyclin H, and MAT1. CAK from the budding yeast *Saccharomyces cerevisiae* was identified as an unusual 44-kilodalton protein kinase, Cak1, that is only distantly related to CDKs. Cak1 accounted for most CAK activity in yeast cell lysates, and its activity was constant throughout the cell cycle. The *CAK1* gene was essential for cell viability. Thus, the major CAK in *S. cerevisiae* is distinct from the vertebrate enzyme, suggesting that budding yeast and vertebrates may have evolved different mechanisms of CDK activation.


The activation of CDKs requires association with a cyclin subunit and phosphorylation by CAK at a conserved threonine residue (*1*). The major CAK activity in vertebrate and starfish cells is a heterotrimer composed of CDK7, cyclin H, and MAT1 (*2*–*4*). The homologous CDK-cyclin complex in fission yeast also has CAK activity (*5*). However, in the budding yeast *Saccharomyces cerevisiae*, the closest CDK7 homolog (Kin28) does not have CAK activity (*6*, *7*), and the enzyme responsible for CDK activation in this organism is unknown.

To explore the nature of CAK in budding yeast, we used conventional chromatographic methods to purify the major CAK activity in yeast lysates (Fig. 1). We measured CAK activity by testing the ability of column fractions to activate the histone H1 kinase activity of purified human CDK2–cyclin A complexes, which are more readily prepared in large quantities than are yeast Cdc28-cyclin complexes. Peak fractions also activated Cdc28-Clb2 (*8*). We estimate that CAK activity was purified over 1000-fold after six chromatographic steps. In the last steps of purification, CAK activity comigrated with a single protein of ~44 kD on polyacrylamide gels (p44) (Fig. 1, B and C). We were unable to purify p44 to homogeneity; however, in multiple preparations, p44 was the only protein that consistently copurified with CAK activity, suggesting that p44 alone was responsible for the activity. This conclusion is supported by results from gel filtration analysis of CAK activity in crude yeast lysates (*8*) or partially purified CAK preparations (Fig. 1A), in which the apparent molecular size of native CAK was ~45 kD.

Tryptic peptides from p44 were subjected to mass spectrometry and amino acid sequencing. Comparison of peptide sequences with the *Saccharomyces* Genomic Database (Stanford University) revealed that the amino acid sequences of two peptides matched predicted sequences in a previously uncharacterized open reading frame, YFL029c, on chromosome VI (*9*). In addition, the masses of these two peptides, as well as those of two additional peptides from p44, matched the theoretical masses of tryptic peptides in the predicted sequence of YFL029c (Fig. 2). The YFL029c open reading frame encodes a protein with sequence similarity to protein kinases and a molecular size of 42,183 daltons. We conclude that this open reading frame encodes p44, which we call Cak1.

Cak1 is only distantly related to other protein kinases. Its closest known relative in any species is yeast Cdc28, with which it shares limited similarity (~23% identity) (Fig. 2). It is even less similar to yeast Kin28 (17% identity) and is therefore not closely related to the CDK7 subfamily. Cak1 is also distinct from most other protein kinases in that it lacks a highly conserved $NH_2$-terminal cluster of glycine residues that contributes to the adenosine triphosphate (ATP) binding site (*10*). Cak1 has large amino acid inserts between conserved kinase subdomains. On the basis of studies of protein kinase structure (*11*, *12*), we predict that these inserts are located in loops between conserved secondary


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structural elements (Fig. 2), away from the catalytic cleft of the kinase.

We constructed a yeast strain in which the genomic Cak1 protein-coding sequence was replaced with a version of Cak1 carrying a COOH-terminal hemagglutinin (HA) epitope tag, allowing detection with antibodies to the tag (*13*). The tagged protein was the only Cak1 protein expressed in this strain and was under the control of the endogenous *CAK1* promoter. Immunoblotting with antibodies to HA (anti-HA) revealed an immunoreactive 44-kD protein in lysates carrying the tagged protein but not in lysates from wild-type cells. Similarly, silver staining of immunoprecipitates with anti-HA from the tagged strain revealed a single 44-kD protein not present in immunoprecipitates from the untagged strain (*8*). We found no evidence for prominent Cak1-associated proteins in immunoprecipitates.

We tested the ability of Cak1 to phosphorylate and activate CDKs in vitro. Immunoprecipitated Cak1 phosphorylated purified human CDK2–cyclin A complexes in vitro, resulting in the activation of their histone H1 kinase activity (Fig. 3A). Cak1 did not phosphorylate or activate a CDK2 mutant in which $Thr^{160}$, the CAK phosphorylation site, was changed to alanine (T160A) (Fig. 3A), confirming that Cak1 phosphorylates CDK2 at the appropriate site. Cak1 did phosphorylate a kinase-deficient CDK2 mutant, indicating that Cak1 does not catalyze CDK2 autophosphorylation (*14*).

Cak1 also phosphorylated purified Cdc28 in vitro (Fig. 3A). Phosphorylation of Cdc28 was not dependent on the presence of cyclin: Cdc28 phosphorylation was equivalent in the presence and absence of the mitotic cyclin Clb2. However, Cak1 activated the histone H1 kinase activity of Cdc28 only when Clb2 was present, confirming that Cdc28 activity is cyclin dependent.

The kinase activity asssociated with Cdc28-Clb2 (Fig. 3A) was lower than that associated with CDK2–cyclin A (note that the kinase reactions in the bottom panel of lanes 7 to 12 contained 10-fold higher quantities of CDK and cyclin proteins than other reactions). Low Cdc28-Clb2 activity is probably due to the low activity of the glutathione-S-transferase (GST)–Clb2 protein, which we have found is highly aggregated on gel filtration. The addition of cyclin A to Cdc28 resulted in high kinase activity similar to that seen with CDK2–cyclin A (Fig. 3A). Limited Clb2 activity may also explain our observations (Fig. 3A) that Cak1 and CDK7 seem equally potent in the activation of Cdc28 despite differences in their ability to phosphorylate Cdc28. Limiting amounts of active Clb2 in these reactions probably restricts kinase activation to a similarly low level.

To verify that Cak1 represents the major CAK activity in *S. cerevisiae*, we immunodepleted Cak1 from cell lysates and measured the amount of CAK activity remaining in the supernatant with human CDK2 or yeast Cdc28 as substrates (Fig. 3B). Cell lysates containing only epitope-tagged Cak1 were incubated with the 12CA5 antibody to the tag, and immune complexes were then adsorbed to protein A–Sepharose and sedimented. Over 97% of CAK activity in the lysate was removed by this procedure (Fig. 3B). In control experiments, immunoprecipitation with 12CA5 did not affect the amount of CAK activity in lysates of wild-type cells in which Cak1 is not tagged. We therefore conclude that Cak1 is the major CAK activity in yeast cell lysates.

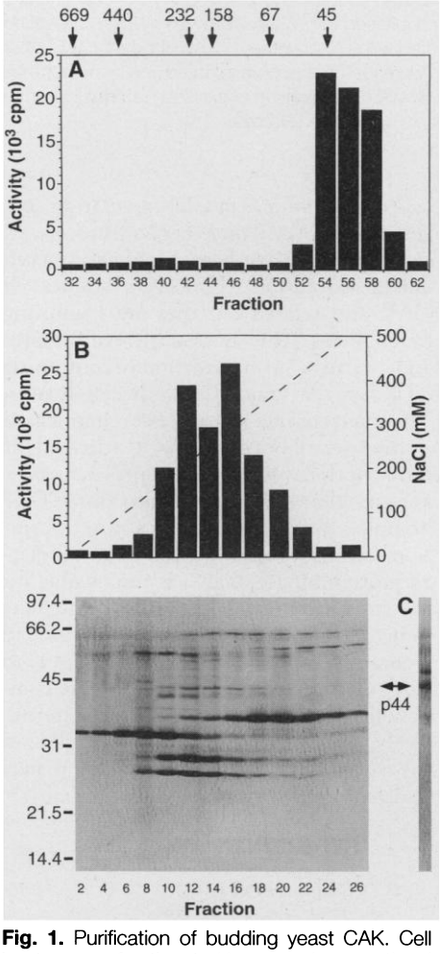


**Fig. 1.** Purification of budding yeast CAK. Cell lysates were fractionated by several conventional chromatographic steps (*20*), and CAK activity in fractions was measured with purified CDK2–cyclin A complexes (*22*). Results from selected steps in two separate preparations are shown. (**A**) CAK after Superdex 200 gel filtration. CAK activity in crude cell lysates migrated at the same size. Positions of molecular size standards (in kilodaltons) are shown (arrows). (**B**) Elution of CAK activity from Cibacron blue Sepharose. (Top) CAK activity; (bottom) silver-stained proteins. Molecular size standards (in kilodaltons) are shown at left. (**C**) Protein components in the peak CAK activity fraction from heparin-Sepharose in a separate preparation. p44 is indicated by ↔.

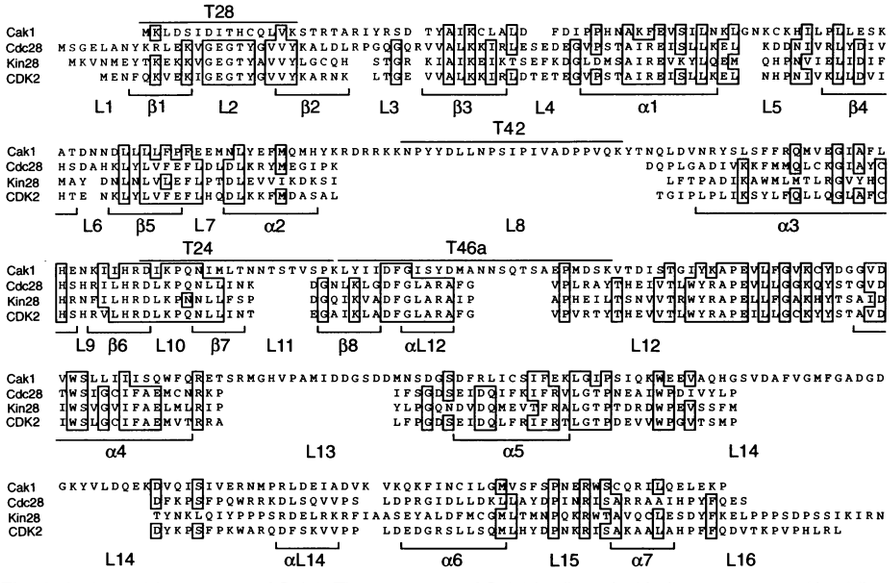


**Fig. 2.** Amino acid sequence of Cak1. The sequence of Cak1 is aligned with the sequences of budding yeast Cdc28, Kin28, and human CDK2. Residues conserved in three or more sequences are boxed and gaps have been introduced to maximize identities. The four tryptic peptides isolated from p44 (*23*) are overlined and numbered. In aligning the Cak1 sequence, we also considered secondary structural elements in protein kinases; these elements in human CDK2 are indicated below the alignment (*12*) (β1 through β8 represent conserved β strands, α1 through α7 represent conserved α helices, and loops between conserved elements are labeled L1 through L16).

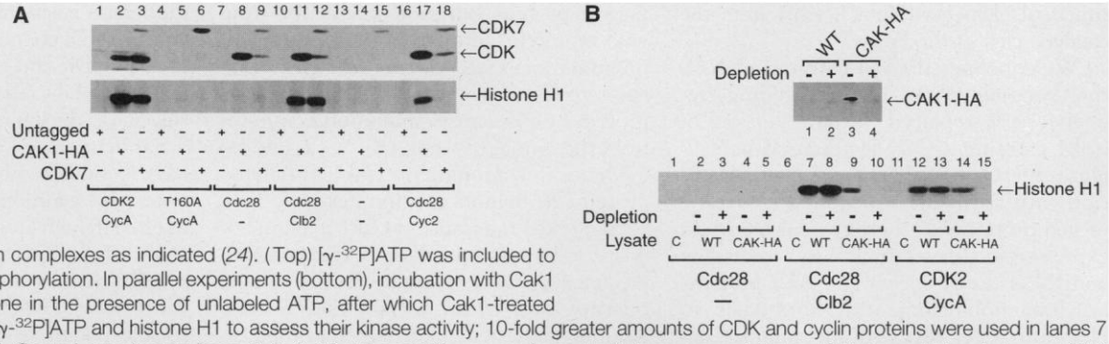


**Fig. 3.** Cak1 is the major CAK activity in yeast cell lysates. (**A**) Anti-HA (12CA5) immunoprecipitates were prepared from lysates of wild-type cells (lanes 1, 4, 7, 10, 13, and 16) or cells expressing only HA-tagged Cak1(lanes 2, 5, 8, 11, 14, and 17) and incubated with ATP and purified CDK-cyclin complexes as indicated (*24*). (Top) [$\gamma$-$^{32}$P]ATP was included to allow detection of CDK phosphorylation. In parallel experiments (bottom), incubation with Cak1 immunoprecipitates was done in the presence of unlabeled ATP, after which Cak1-treated CDKs were incubated with [$\gamma$-$^{32}$P]ATP and histone H1 to assess their kinase activity; 10-fold greater amounts of CDK and cyclin proteins were used in lanes 7 to 12 than in other lanes (*24*). Control experiments (lanes 3, 6, 9, 12, 15, and 18) were performed with immunoprecipitates of HA-tagged trimeric human CAK (CDK7–cyclin H–MAT1); in these reactions, the CDK7-HA subunit was also phosphorylated. Apparent discrepancies between cyclin A–Cdc28 phosphorylation and activation (lanes 16 to 18) were not reproducible; quantitation of results from this and other experiments indicates that phosphorylation and activation increase in parallel. Purified CDK-cyclin complexes were prepared as described (*25*). (**B**) (Top) Lysates of wild-type (lanes 1 and 2) and Cak1-HA cells (lanes 3 and 4) were immunodepleted with 12CA5 (lanes 2 and 4), and remaining Cak1-HA was detected by immunoprecipitation with 12CA5 followed by immunoblotting (*26*). (Bottom) Lysates from wild type (WT) or cells expressing only HA-tagged Cak1 (CAK-HA) were incubated with (+) or without (−) antibody 12CA5. After removal of immune complexes, samples were incubated with the indicated CDK-cyclin complexes, and CDKs were then immunoprecipitated and tested for their ability to phosphorylate histone H1 (*26*). In control experiments (C), CDKs were incubated with buffer alone. Scintillation counting of excised histone H1 bands indicated that the amount of CAK activity remaining after depletion (lanes 10 and 15) was <3% of the activity in undepleted lysates.

Monomeric Cak1 protein appears to be responsible for the CAK activity we observe. Cak1 migrates on gel filtration as a monomer, and we did not observe any prominent comigrating proteins during CAK purification or in Cak1-HA immunoprecipitates. We also expressed CAK as a 6-histidine–tagged fusion protein in *Escherichia coli*, and purification of this protein by metal affinity and gel filtration chromatographies revealed that the protein was monomeric and had CAK activity (*8*). We therefore think it likely that Cak1, unlike the CDKs, does not require an activating subunit.

We analyzed the activity of Cak1 at different stages of the cell cycle. Cells carrying tagged Cak1 were arrested in $G_1$ by treatment with the mating pheromone $\alpha$ factor. The pheromone was then washed out, allowing synchronous progress through S phase and mitosis. Immunoblotting analysis of the mitotic cyclin Clb2 was used to estimate the time of mitosis (Fig. 4C). Cak1 kinase activity and the amount of Cak1 protein (Fig. 4, A and B) were constant throughout the cell cycle, like those of CAK in higher eukaryotes (*15*).

To clarify the physiological role of Cak1, we determined the phenotypic consequences of deleting the *CAK1* gene. A diploid strain was constructed in which one chromosomal copy of the *CAK1* coding region was completely replaced with the *TRP1* gene. Analysis of spores produced by this diploid indicated that *CAK1* is essential for cell viability (*8*). Spores bearing the *CAK1* deletion formed microcolonies containing between 8 and 12 cells with a terminal phenotype that was heterogeneous: 21% of cells in these colonies were unbudded, 5% had small buds, 33% had large buds, and the remainder exhibited abnormal morphology. If the sole function of Cak1 were to activate Cdc28, then one might predict that loss of Cak1 function would yield a uniform cell cycle arrest similar to that observed in temperature-sensitive cdc28 mutants. However, dephosphorylation of Cdc28 as a result of gradual Cak1 depletion might result in a more heterogeneous arrest phenotype. Alternatively, Cak1 may have additional functions in the activation of other CDKs such as Kin28 or Pho85.

Previous studies of CDK regulation suggest that fundamental mechanisms of cell cycle control have been highly conserved during evolution. However, studies of CAK and related enzymes are beginning to challenge this notion. In vertebrates and starfish, a heterotrimer containing CDK7 is the major CAK in cell lysates. The same enzyme has also been implicated in the control of polymerase II–dependent transcription (*4*, *16*). In contrast, it now appears that in *S. cerevisiae* the CDK7 homolog, Kin28, participates in transcriptional control (*6*, *17*), whereas an unrelated protein kinase, Cak1, is responsible for the activation of CDKs. This finding may indicate that budding yeast have evolved separate components, Kin28 and Cak1, to carry out functions served by a single component, CDK7, in vertebrates. Alternatively, CDK7 may not be the sole CAK in the vertebrate cell; CDK activation may also require a vertebrate Cak1 homolog.

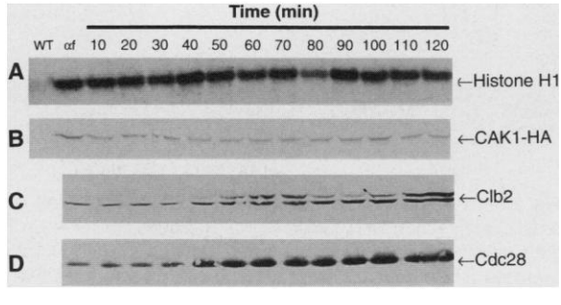


**Fig. 4.** Constant amounts of Cak1 protein and activity throughout the cell cycle. Cak1-HA cells were arrested in $G_1$ by treatment with $\alpha$ factor ($\alpha$f), washed to allow release from the arrest, and lysed at the indicated times (*21*). Lysates were also prepared from asynchronous wild-type cells (WT). (**A**) Cak1 activity in 12CA5 immunoprecipitates, measured by activation of Cdc28-Clb2 complexes (*24*). (**B** to **D**) Immunoblots with antibodies to the HA epitope tag (B), Clb2 (C), and Cdc28 (D). On the Clb2 immunoblot, the upper band in the doublet is Clb2; the lower band is a nonspecific band. Immunoblots were done as described (*21*).

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13. K699 cells were transformed with the CAK1-HA integration vector, pFHE86, containing an 860–base pair fragment of the Cak1 coding region (derived from polymerase chain reaction amplification of genomic DNA) fused to an HA tag (*18*) followed by an actin terminator, in a vector (pRS305) containing the *LEU2* gene [R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)]. The COOH-terminal Cak1 sequence was thereby changed from EKP to EKEMAYPYDVPDYASLGPGL (*19*).
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19. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; K, Lys; L, Leu; M, Met; P, Pro; S, Ser; V, Val; and Y, Tyr.
20. In a typical CAK preparation, log phase cells of strain K699 were lysed by mechanical disruption in a Bead Beater (BioSpec) in lysis buffer [25 mM Hepes (pH 7.4), 50 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM dithiothreitol (DTT), 5 mM NaF, 5 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), and aprotinin (9 mU/ml)]. Nucleic acids were precipitated with polyethyleneimine, and the lysate was clarified by centrifugation (1 hour, 100,000*g*). CAK activity in the crude lysate (~11 g of total protein) was precipitated with ammonium sulfate (30 to 60% saturation), resuspended in buffer D [25 mM Hepes (pH 7.4), 1 M $(NH_4)_2SO_4$, 10% glycerol, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 5 mM β-glycerophosphate], and loaded onto a 180-ml phenyl-Sepharose column. The column was washed with buffer A [25 mM Hepes (pH 7.4), 10% glycerol, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 5 mM β-glycerophosphate] plus 300 mM $(NH_4)_2SO_4$ and then eluted with buffer A. The eluate was loaded on an 80 ml DEAE-Sepharose column and eluted with a linear NaCl gradient in buffer A. Peak fractions were passed through sulphopropyl (SP) Sepharose, loaded onto Cibacron blue Sepharose (8 ml), and eluted with an NaCl gradient in buffer A. Peak fractions were concentrated by ammonium sulfate precipitation and fractionated on a Pharmacia Superdex 200 gel filtration column (125 ml) equilibrated with buffer A (Fig. 1A). Peak fractions were pooled and loaded onto a heparin-Sepharose column (25 ml) and eluted isocratically with buffer A. The eluate was concentrated with a 1-ml Mono Q column, loaded onto a 1-ml Cibacron blue Sepharose column, and eluted with a linear NaCl gradient (Fig. 1B). Figure 1C illustrates the peak CAK fraction from a heparin-Sepharose column in a separate preparation.
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22. For measurement of CAK activity, purified complexes of human CDK2–HA and a truncated form of human cyclin A (CycAΔ171) (*3*) were immobilized on protein A–Sepharose beads coated with monoclonal antibody to HA (12CA5) and incubated for 15 min at 25°C with the desired sample and 10 mM $MgCl_2$ and 1 mM ATP. Beads were washed once with HBST [10 mM Hepes (pH 7.4), 150 mM NaCl, 0.05% Triton X-100], once with HBS [10 mM Hepes (pH 7.4), 150 mM NaCl], and once with kinase buffer [25 mM Hepes (pH 7.4), 10 mM $MgCl_2$], and then incubated for 10 min at 25°C with kinase buffer plus 100 μM ATP, 5 μg histone H1, and 1 μCi of [γ-$^{32}$P]ATP.
23. Partially purified CAK was precipitated with acetone, subjected to polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The 44-kD component (~300 to 500 ng) was excised, digested with trypsin, and fractionated by reversed-phase high-performance liquid chromatography on a 1.0-mm Reliasil $C_{18}$ column. The masses of four tryptic peptides were determined by matrix-assisted laser-desorption time-of-flight mass spectrometry [H. Erdjument-Bromage, M. Lui, D. M. Sabatini, S. H. Snyder, P. Tempst, *Protein Sci.* **3**, 2435 (1994); S. Geromanos, P. Casteels, C. Elicone, M. Powell, P. Tempst, in *Techniques in Protein Chemistry*, J. W. Crabb, Ed. (Academic Press, San Diego, CA, 1994), vol. 5, pp. 143–150], and two of these peptides were sequenced by automated Edman degradation [P. Tempst, S. Geromanos, C. Elicone, H. Erdjument-Bromage, *Methods* **6**, 248 (1994)]. Small amounts of two peptides from unrelated proteins were also present in the 44-kD fraction and presumably represent contaminants.
24. Yeast lysates were prepared by mechanical disruption in lysis buffer (*20*). Lysate (500 μg) or purified mammalian CAK (CDK7-HA–cyclin H–MAT1, 100 ng) were subjected to immunoprecipitation with 12CA5 as described (*21*). To analyze CDK phosphorylation, we incubated immunoprecipitates for 10 min at 25°C in kinase assay buffer (*22*) with 5 μCi of [γ-$^{32}$P]ATP and 0.5 μg of each CDK-cyclin pair (except that 1.5 μg of GST-Clb2 was used for Clb2 experiments). To analyze CDK activation, we incubated immunoprecipitates for 15 min at 25°C with 500 ng of each CDK-cyclin pair (or 1.5 μg of GST-Clb2) and 10 mM $MgCl_2$, 1 mM ATP, and 5 μg of HA peptide to reduce binding of HA-tagged substrates to 12CA5 on the beads. To analyze histone H1 activity, we incubated 5 ng (lanes 1 to 6, and 16 to 18) or 50 ng (lanes 7 to 12) of the indicated cyclin-CDK pair for 10 min at 25°C with kinase buffer plus 5 μg of histone H1 and 1 μCi of [γ-$^{32}$P]ATP.
25. Purified CDK2-HA, CDK2(T160A)-HA, and CycAΔ171 were prepared as described (*3*, *18*). Cdc28-HA and the mammalian CAK trimer were expressed in Sf9 insect cells with recombinant baculoviruses and purified by conventional chromatography. A GST-Clb2 fusion protein was expressed in bacteria (*21*) and purified with glutathione affinity resin followed by Hi-Trap Q-Sepharose.
26. For immunodepletion experiments, 50 μg of 12CA5 and 100 μl of protein A–Sepharose were added to 500 μg of lysate, incubated for 1 hour at 4°C, and removed by centrifugation. Control lysates were incubated with beads alone. To analyze Cak1 protein remaining after depletion, we immunoprecipitated 500 μg of control or immunodepleted lysate with 12CA5 and analyzed the immunoprecipitates by immunoblotting with antibody to HA (16B12). To assess CAK activity remaining after depletion, we incubated 100 ng of each CDK-cyclin complex for 15 min at 25°C with 50 μg of lysate (or lysis buffer) and 10 mM $MgCl_2$ and 1 mM ATP. Activated CDKs were recovered by immunoprecipitation with 12CA5 and tested for histone H1 kinase activity as described (*22*).
27. We thank R. Fisher, H. Chamberlin, and D. Kellogg for reagents; A. Murray for valuable advice; K. Liu and members of the Morgan laboratory for comments on the manuscript; and M. Lui for assistance with protein structural analysis. Supported by grants (to D.O.M.) from the National Institute of General Medical Sciences, the Markey Charitable Trust, and the Rita Allen Foundation, as well as grants from the NSF (to P.T.) and the National Cancer Institute (to the Sloan-Kettering Sequencing Lab). F.H.E. is supported by a University of California President's fellowship. A.F. is supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada.

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# Regulation of Interferon-γ–Activated STAT1 by the Ubiquitin-Proteasome Pathway

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STAT proteins (signal transducers and activators of transcription) are latent cytoplasmic transcription factors that are phosphorylated by Janus kinases in response to cytokines. Phosphorylated STAT proteins translocate to the nucleus, where they transiently turn on specific sets of cytokine-inducible genes. The mechanism that controls the amounts of activated STAT proteins is not understood. STAT1 proteins activated by interferon-γ treatment in HeLa cells were shown to be stabilized by a proteasome inhibitor and ubiquitinated in vivo. Thus, the amount of activated STAT1 may be negatively regulated by the ubiquitin-proteasome pathway.

Cytokines regulate many aspects of cellular growth, differentiation, and activation and play a critical role in immune and inflammatory responses. Analysis of the pathways involved in interferon-α (IFN-α) and IFN-γ induction has led to the identification of the Janus kinase (Jak)–STAT pathway of cytokine induction (*1*). The binding of IFNs to their corresponding cell surface receptors results in the activation of Jak kinases, which phosphorylate STAT proteins. In the case of IFN-γ, phosphorylation of the STAT1 protein leads to STAT1's translocation into the nucleus, where it activates specific target genes by binding to a regulatory sequence termed GAS (IFN-γ–activated site). The amount of phosphorylated STAT1 protein is maximal between 15 and 30 min after IFN-γ treatment and then rapidly decreases to undetectable levels within 1 to 2 hours (*2*, *3*). This negative regulation of STAT levels is very important because the duration of the IFN response is critical and cells must respond to numerous cytokines simultaneously. Without this regulation, toxic amounts of gene products could accumulate, and cells could not modulate or coordinate various cytokine responses. In this report, we address the possible role of ubiquitin-dependent proteolysis

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