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SCF^{Cdc4}-mediated Degradation of the Hac1p Transcription Factor Regulates the Unfolded Protein Response in *Saccharomyces cerevisiae*[D]

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The *Saccharomyces cerevisiae* basic leucine zipper transcription factor Hac1p is synthesized in response to the accumulation of unfolded polypeptides in the lumen of the endoplasmic reticulum (ER), and it is responsible for up-regulation of ~5% of all yeast genes, including ER-resident chaperones and protein-folding catalysts. Hac1p is one of the most short-lived yeast proteins, having a half-life of ~1.5 min. Here, we have shown that Hac1p harbors a functional PEST degron and that degradation of Hac1p by the proteasome involves the E2 ubiquitin-conjugating enzyme Ubc3/Cdc34p and the SCF^{Cdc4} E3 complex. Consistent with the known nuclear localization of Cdc4p, rapid degradation of Hac1p requires the presence of a functional nuclear localization sequence, which we demonstrated to involve basic residues in the sequence $_{29}$RKRAKTK$_{35}$. Two-hybrid analysis demonstrated that the PEST-dependent interaction of Hac1p with Cdc4p requires Ser146 and Ser149. Turnover of Hac1p may be dependent on transcription because it is inhibited in cell mutants lacking Srb10 kinase, a component of the SRB/mediator module of the RNA polymerase II holoenzyme. Stabilization of Hac1p by point mutation or deletion, or as the consequence of defects in components of the degradation pathway, results in increased unfolded protein response element-dependent transcription and improved cell viability under ER stress conditions.

INTRODUCTION

In eukaryotic cells, the endoplasmic reticulum (ER) is the portal for newly synthesized proteins destined for all compartments of the exocytic and endocytic pathways as well as for the cell surface and the extracellular space. Transport along the exocytic pathway requires that passenger proteins are correctly folded and assembled (Gething *et al*., 1986; for review, see Ellgaard and Helenius, 2003), a process that is assisted by molecular chaperones and folding catalysts resident in the ER lumen (for review, see van Anken and Braakman, 2005). The concentrations of these components in the ER are adjusted according to need by the unfolded protein response (UPR; Kozutsumi *et al*., 1988; Mori *et al*., 1992; for review, see Kaufman, 1999; Ma and Hendershot, 2001; Patil and Walter, 2001; Schröder and Kaufman, 2005). The UPR regulates the transcription of ~5% of the open reading frames in the *Saccharomyces cerevisiae* genome, many of which encode proteins with functions involved in diverse processes, including protein translocation, glycosylation, folding and degradation, lipid/inositol metabolism, vesicular trafficking, vacuolar protein sorting, and cell wall biogenesis (Travers *et al*., 2000).

The yeast unfolded protein response (UPR) involves two unique participants, the Ire1p transmembrane receptor kinase/endonuclease (Cox *et al*., 1993; Mori *et al*., 1993), and the basic leucine zipper (bZip) transcription factor Hac1p (Cox *et al*., 1996; Mori *et al*., 1996), which transactivates genes bearing UPRE elements (Mori *et al*., 1992, 1998; Patil and Walter, 2004). *HAC1* mRNA is synthesized constitutively as a precursor bearing a 252-nucleotide intron that blocks translation as the result of base pairing with a sequence in the 5′-untranslated region of the mRNA (Chapman and Walter, 1997; Kawahara *et al*., 1997; Ruegsegger *et al*., 2001). This intron is removed by the endoribonuclease activity of the C terminus of Ire1p (Cox *et al*., 1996; Kawahara *et al*., 1997; Sidrauski and Walter, 1997), which is activated after oligomerization of the receptor in response to the accumulation of unfolded proteins in the ER and *trans*-autophosphorylation of the kinase domain (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). The resulting *HAC1* mRNA exons are joined by the Rlg1p ligase to produce the mature, efficiently translated mRNA (Sidrauski *et al*., 1996; Kawahara *et al*., 1997). This process brings together sequences in the first exon that encode a potential nuclear localization signal and the DNA binding domain with sequences in the second exon that encode the transcriptional activator domain (TAD) (Mori *et al*., 2000).

Previous studies on the regulation of the UPR have largely focused on events that initiate the synthesis of Hac1p. However, mechanisms that regulate the rate of turnover of Hac1p

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Table 1. Yeast strains

Strain	Genotype	Reference or source
W303-1a	*MAT*a *his3-11 leu2-3 ura3-1 ade2-1 trp1-1 can1-100*	B. Futcher
can1-1	W303 but *can1-1*	Ghislain *et al.* (1993)
DL376	*MATa his4 leu2-3,112 ura3-52 trp1-1 can1^r pkc1*Δ*::LEU2*	Levin and Bartlett-Heubusch (1992)
DL455	*MATa his4 leu2-3,112 ura3-52 trp1-1 can1^r mpk1*Δ*:TRP1*	Lee *et al.* (1993)
DL1783	*MAT*a *his4 leu2-3,112 ura3-52 trp1-1 can1^r*	Lee and Levin (1992)
JN1	*MAT*α *ura3, his3, leu2, lys2, trp1, gal4, GAL1-LacZ reporter*	Muratani *et al.* (2005)
JN5	JN1 but *srb10*Δ*::kanMX6*	Muratani *et al.* (2005)
KMY1005	*MAT*α *leu2-3,112 ura3-52 his3-*Δ*200 trp-901 lys2-801*	Mori *et al.* (1996)
KMY1045	KMY1005 but *hac1*Δ*::TRP1*	Mori *et al.* (1996)
KMY2005	*MAT*α *leu2-3,112 ura3-52 his3*Δ*200 trp1*Δ*901 lys2-801 sec53-6 gal1 suc2*	Mori *et al.* (1996)
KMY2105	*MAT*α *leu2-3,112 ura3-52 his3*Δ*200 trp1*Δ*901 lys2-801 sec53-6 gal1 suc2* UPRE-lacZ::URA3	Kawahara *et al.* (1997)
KMY2145	KMY2105 but *hac1*Δ*::TRP1*	Kawahara *et al.* (1997)
L40	*MAT*α *leu2-3,112 ura3-1 his3-*Δ*200 15 trp1-901 ade2-1 lys2-801am URA3::(lexAOP)8-lacZ LYS2::(lexAop)4-HIS3*	Hollenberg *et al.* (1995)
LHY100	KMY2105 but *mpk1*Δ*::HIS3*	This study
LHY102	KMY2005 but *pkc1*Δ*::LEU2*	This study
MT668*	W303 but *cdc4-1*	Patton *et al.* (1998)
MT670	W303 but *cdc34-1*	Willems *et al.* (1996)
MT871	W303 but *cdc53-1*	Willems *et al.* (1996)
NCY1810	DL1783 but *hac1*Δ*::kanMX6*	This study
NCY1811	KMY1045 but *srb10*Δ::His3MX6*1^r*	This study
PY1	15Daub(wt): a *bar1*Δ *ura3*Δ*ns ade1 his2 leu2-3,112 trp1-1*	Kaiser *et al.* (2000)
PY283*	PY1 but *met30-6::KAN^R*	Kaiser *et al.* (2000)
Y80	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Bai *et al.* (1996)
Y552	Y80 but *skp1-11*	Bai *et al.* (1996)
Y554	Y80 but *skp1-12*	Bai *et al.* (1996)
BY4742	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 can1-100*	Research Genetics
RG16902*	BY4742 but *grr1*Δ*::kanMX6*	Research Genetics
RG12708*	BY4742 but *hrt3*Δ*::kanMX6*	Research Genetics
RG11133*	BY4742 but *skp2*Δ*::kanMX6*	Research Genetics
RG11856*	BY4742 but *dia1*Δ*::kanMX6*	Research Genetics
RG11597*	BY4742 but *rax1*Δ*::kanMX6*	Research Genetics
RG13343*	BY4742 but *cos111*Δ*::kanMX6*	Research Genetics
RG14065*	BY4742 but locus YDR131C deleted with *kanMX6*	Research Genetics
RG14173*	BY4742 but locus YLR224W deleted with *kanMX6*	Research Genetics
RG11276*	BY4742 but locus YJL149W deleted with *kanMX6*	Research Genetics
RG13578*	BY4742 but locus YDR219C deleted with *kanMX6*	Research Genetics
RG11221*	BY4742 but *rcy1*Δ*::kanMX6*	Research Genetics
RG14611*	BY4742 but *rtf1*Δ*::kanMX6*	Research Genetics
RG13072*	BY4742 but locus YBL046W deleted with *kanMX6*	Research Genetics
RG13665*	BY4742 but locus YDR306C deleted with *kanMX6*	Research Genetics
RG12298*	BY4742 but *rev7*Δ*::kanMX6*	Research Genetics
RG17172*	BY4742 but locus YBR280C deleted with *kanMX6*	Research Genetics
RG15277*	BY4742 but *flm1*Δ*::kanMX6*	Research Genetics
RG15261*	BY4742 but locus YLR352W deleted with *kanMX6*	Research Genetics
RG11982*	BY4742 but *ela1*Δ*::kanMX6*	Research Genetics
RG15308*	BY4742 but *bdf1*Δ*::kanMX6*	Research Genetics
BY4739	*MAT*α *leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	Research Genetics
RG10482*	BY4739 but *ufo1*Δ*::kanMX6*	Research Genetics

* F-box mutants.

will also be crucial in determining the cellular concentration of the active transcription factor and thus the magnitude of the stress response. The concentration of Hac1p should also affect the scope of the response, because different classes of UPR-regulated genes are transactivated at different threshold levels of Hac1p (Leber *et al*., 2004). The rate of degradation of Hac1p will also determine how quickly the response is terminated once the ER stress is removed. In this study, we have investigated the sequence elements and cellular machinery that contribute to the very rapid rate of turnover of Hac1p ($t_{1/2}$ of 1–2 min; Kawahara *et al*., 1997; Chapman and Walter, 1997).

MATERIALS AND METHODS

Strains and Media

The yeast strains used in this study are listed in Table 1. Cells were grown in YP medium containing glucose (YPD) or synthetic complete media lacking appropriate amino acids (Kaiser *et al*., 1994). Strains containing a temperature-sensitive mutation were grown at the permissive temperature (23°C) before incubation under nonpermissive conditions (37°C). The NCY1810 Δ*hac1* strain (DL1783, *hac1*Δ*::kanMX6*) was generated by direct replacement of the *HAC1* open reading frame with the kanMX6 cassette (Guldener *et al*., 1996). The NCY1811 Δ*hac1* Δ*srb10* strain (KMY1045, *srb10*Δ::*His3MX6*) was generated by direct replacement of the *SRB10* open reading frame with His3MX6 cassette as described by Longtine *et al*. (1998). LHY100 (KMY2105 *mpk1*Δ*::HIS3*) was constructed by single-step gene replacement (Rothstein, 1991) by using the *HIS3*

gene amplified from plasmid pRS313 (Sikorski and Hieter, 1989) with primers incorporating the first and the last 50 nucleotides of the *MPK1* open reading frame. LHY102 (KMY2005 *pkc1Δ::LEU2*) was also constructed by single step gene replacement by using plasmid pL924 (Levin *et al*., 1990). In all cases, clones containing the desired deletion were obtained after growth on the appropriate selective medium. The genotypes were confirmed by polymerase chain reaction (PCR).

Plasmid Constructions and Oligonucleotide-directed Mutagenesis

All plasmid manipulations were carried out using standard protocols (Sambrook *et al*., 1989). References to the sources or details of the construction of the various plasmids used in this study can be found in the Supplemental Material. Descriptions of the procedures used to make *HAC1* deletion and point mutants can also be found in the Supplemental Material, and the oligonucleotides used in this work are listed in Supplemental Table S1. DNA sequence analysis was used to confirm the introduction of mutations and to rule out the possibility that additional alterations had been introduced by the mutagenesis or cloning procedures.

PESTfind Analysis of Hac1p Sequence

The PESTfind server (emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind) was used to identify possible PEST sequences within Hac1p.

Protein Synthesis Shutoff Assay

S. cerevisiae cells were grown overnight to OD_{600} 0.6 at 30 or 23°C (for temperature-sensitive yeast strains) in 50 ml of YPD media or SC-Leu, or SC-Trp-Ura. To examine the turnover of Hac1p, tunicamycin (Calbiochem. San Diego, CA) was added to a final concentration of 5 μg/ml to induce the UPR, and the culture was incubated for another 90 min. In temperature-sensitive yeast strains, cultures were shifted from 23°C to the nonpermissive temperature of 37°C for 30 min. An 8-ml aliquot was removed as the zero-time sample and transferred to a tube containing 100 μl of 20% sodium azide (Sigma-Aldrich, St. Louis, MO) and 1 ml of dimethyl sulfoxide (Sigma-Aldrich), mixed by inversion, and snap-frozen in liquid nitrogen. Cycloheximide (Sigma-Aldrich) was added to a final concentration of 1 mg/ml to the bulk culture to halt protein synthesis, and incubation was continued. Samples were collected at various times between 1 and 30 min after the addition of cycloheximide and snap-frozen as described above. Samples were thawed in a 4°C water-bath (~45 min) before pelleting at 4°C (10 min; 3000 rpm) and storage at −80°C. To examine the turnover of Ura3/HAp fusion proteins, the zero-time point sample was collected before adding cycloheximide and aliquots were collected at 30-, 60-, 90-, and 150-min time points.

Cell Extracts and Immunoblotting

Unless otherwise indicated protein extracts from yeast cells were made in EZ buffer (60 mM Tris, pH 6.8, 10% [vol/vol] glycerol, 2% [wt/vol] SDS, and 5% [vol/vol] β-mercaptoethanol) by boiling for 10 min, followed by mixing and centrifugation (13,000 rpm for 10 min) (Muratani *et al*., 2005). Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad, Hercules, CA). Volumes of extract containing equal amounts of total protein (~80 μg) were boiled in sample buffer for 10 min and then loaded on either an 8 or 12% SDS gel and submitted to polyacrylamide gel electrophoresis (PAGE). For immunoblot analysis, the proteins were transferred onto nitrocellulose (Protran; Whatman Schleicher and Schuell, Keene, NH) by wet transfer in the Mini Trans-Blot Cell (Bio-Rad) and blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Detection of wild-type Hac1p and the various mutant Hac1p proteins was performed using a polyclonal anti-Hac1p antibody (Kawahara *et al*., 1997) or an anti-$Hac1p^{i}$ tail antibody (Cox and Walter, 1996). Analysis of Ura3/HA and Hac1/HA fusion proteins used anti-hemagglutinin (HA) tag antibodies, (Muratani *et al*., 2005), whereas detection of green fluorescent protein (GFP) fusion proteins used polyclonal anti GFP antibodies (a gift from Pamela Silver, Dana-Farber Institute, Boston, MA). Subsequently, the membranes were probed with monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (a gift from Trevor Lithgow, University of Melbourne, Melbourne, Australia) as a control for protein loading. The proteins were detected using enhanced chemiluminescence reagents (Pierce Chemical, Rockford, IL or Roche Diagnostics, Indianapolis, IN) according to the manufacturers' instructions. Densitometry was carried out using ImageQuant 4.5 software (Media Cybernetics, Silver Spring, MD).

Growth Assays

Yeast cells were grown at 30°C in appropriate media until OD_{600} reached 0.6. Cultures were then diluted with water to OD_{600} of 0.04, and a series of 1:10 serial dilutions in water were prepared. For each dilution, a 5-μl aliquot was spotted onto solid media (YPD and YPD supplemented with either 0.5 or 1.0 μg/ml tunicamycin for ER stress survival assays, and SC lacking tryptophan or SC lacking both tryptophan and uracil for uracil prototrophy assays) and incubated at 30°C until colonies formed (2–3 d).

Fluorescence Microscopy

Yeast cells expressing GFP fusion proteins were prepared for visualization as follows: log-phase cultures (OD_{600} of 1.0–1.5) were grown at 30°C in synthetic medium in the absence of methionine to induce expression from the *MET25* promoter of the pGFP-Nfus vector. Cells were fixed by adding 1/10 volume formaldehyde (standard stock solution is 37%) directly to the medium and by incubating for at least 30 min. Cells were harvested and washed twice with 0.1 M potassium phosphate, pH 7.5, and then twice with 1× phosphate-buffered saline (PBS), and then resuspended in PBS (200 μl per 10 ml of culture). Coverslips were coated with poly-L-lysine (Sigma-Aldrich) and allowed to dry after which 20 μl of the cell suspension was smeared evenly on the coverslip and allowed to dry. The coverslip was then inverted onto a slide with 10 μl of Mowiol (Calbiochem) containing 4,6-diamidino-2-phenylindole (DAPI) dye (Sigma-Aldrich). Cells were viewed at room temperature with an Axioplan2 microscope (Carl Zeiss, Thornwood, NY) equipped with an AxioCam Mrm digital camera. Picture analysis was performed using Axiovision2 software.

Two-Hybrid Analysis

Yeast two-hybrid assays were carried out using pACT encoding the Gal4 transcriptional activation domain (AD) alone as a negative control or Gal4AD fused with *CDC4* sequences encoding residues (339-779) (Drury *et al*., 1997) as bait to test the interaction of wild-type and mutant Hac1p sequences with Cdc4p substrates. pBTM116 and pBTM116-CDC6(1–47), which encode the LexA DNA binding domain (BD) alone or fused to residues 1–47 of Cdc6p (Drury *et al*., 1997), were used as negative and positive prey plasmids, respectively. pBTM116–HAC1 fusion constructs were generated as described in the Supplemental Material. pACT and pBTM116 constructs were transformed into *S. cerevisiae* strain L40 (Hollenberg *et al*., 1995). Protein–protein interaction assays were performed by growing transformants on nitrocellulose filters before lysing the cells and measuring β-galactosidase activity as described by Xie *et al*. (1993).

Osmotic and UPR Stress Treatments

For experiments using osmotic modulation, cells were grown to mid-logarithmic phase (OD_{600} of 0.8–1.0) at 23°C (for KMY2015 *sec53* cells) or 30°C (for $SEC53^{+}$ cells) in either YPD or selective media supplemented with 1 M sorbitol. To apply osmotic stress, cell cultures were diluted with 4 parts of osmotic diluents: 0.4% glucose (hypotonic) or 0.4% glucose + 1M sorbitol (isotonic) made up in water or selective medium and prewarmed to the temperature of the subsequent incubation. Where appropriate, tunicamycin was added to the diluents to a final concentration of 5 μg/ml to initiate UPR stress. For *sec53* cells, ER stress was imposed either by incubation at the semipermissive temperature of 30°C or by combining incubation at 30°C with tunicamycin treatment. Control cells (no ER stress) were incubated at 23°C (*sec53* cells) or at 30°C ($SEC53^{+}$ cells) in the absence of tunicamycin. At the end of each incubation period cells were placed on ice, pelleted, and frozen in liquid N_2. All further manipulations were done at 0–4°C.

β-Galactosidase Assay

Cells were lysed using glass beads and the extracts assayed for β-galactosidase activity as described previously (Kaiser *et al*., 1994). Protein concentrations were determined using the Bio-Rad DC protein assay kit, and β-galactosidase activity was defined as units per milligram of protein where 1 U results in OD_{420} of 0.001/min.

In Vivo Labeling and Immunoprecipitation

^{35}S Labeling and Immunoprecipitation of Hac1p. Yeast cells were grown to OD_{600} of 0.5–0.8 at 23°C. Cells were resuspended in low sulfate medium at a density of 3 OD_{600} units/ml and incubated for 30 min at 23°C before 4-ml aliquots were taken for incubation at 23°C (control) or 30°C (ER stress) for a further 60 min, at which point they were pulse labeled with 150 μCi of [^{35}S]Promix labeling mix (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 5 min and harvested as described previously (Kawahara *et al*., 1997). Cell extracts were prepared as described previously (Cox *et al*., 1997), and equal amounts of protein (70–100 μg) were taken for immunoprecipitation (IP) in a total volume of 500 μl. IP reactions were precleared using preimmune serum and 50 μl of 10% (vol/vol) Pansorbin (Calbiochem), and Hac1p was precipitated with 5 μl of anti-Hac1p antiserum and 50 μl of protein A-Sepharose (50%, vol/vol). Washings followed established protocols (Franzusoff *et al*., 1991). Hac1p was eluted from beads in Laemmli loading buffer and separated by SDS-PAGE. After treatment with Amplify (GE Healthcare), gels were dried and subjected to fluorography.

^{32}P Labeling and Immunoprecipitation of Hac1p. Yeast cells were grown in high phosphate medium to OD_{600} of 0.8 at 23°C, washed into low phosphate medium at a density of OD_{600} of 0.8, and incubated at 23°C for a further 4 h (Shamu and Walter, 1996). The OD_{600} was adjusted to 0.8, and 4 ml of cells was labeled with 100 μCi of [^{32}P]orthophosphoric acid/ml at 23°C for 1 h, and then the cells were divided into two aliquots and placed at either 23 or 30°C for a further 2 h. Cells were harvested in the presence of phosphatase

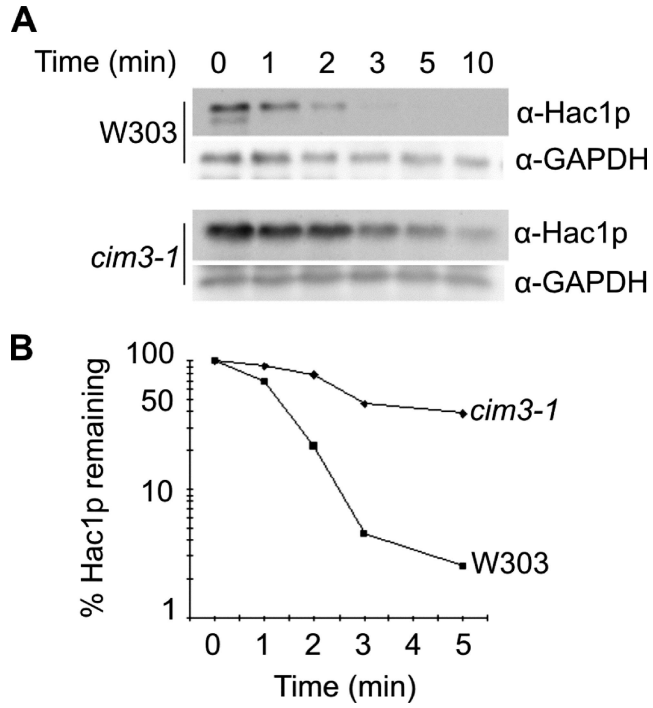

Figure 1. Hac1p stability is regulated via the 26S proteasome. (A) *cim3–1* cells and parental W303 cells were grown to OD_{600} of 0.6 at 23°C in YPD media. The UPR stressor tunicamycin was then added to a final concentration of 5 μg/ml to induce synthesis of Hac1p, and incubation was continued for 90 min before the cultures were shifted to the nonpermissive temperature of 37°C. Cycloheximide (final concentration 1 mg/ml) was added to the culture to halt protein synthesis, and samples were collected at the indicated chase times, snap-frozen and treated as described in *Materials and Methods*. Cell extracts were then prepared and protein concentrations were measured. Samples containing 80 μg of cellular protein were analyzed by SDS-PAGE and immunoblotted first with α-Hac1p then with α-GAPDH antibodies. (B) The plots were generated from the data shown in A by using ImageQuant 4.5 software.

inhibitors (including 50 nM calyculin A; Shamu and Walter, 1996) and extracts were prepared (Cox *et al*., 1993). Volumes of cell lysates were adjusted to 160 μl, of which 100 μl was used in each IP reaction in a total volume of 500 μl with the addition of phosphatase inhibitors (Shamu and Walter, 1996). The concentration of protein in the lysates was determined using the Bio-Rad DC protein assay kit. After immunoprecipitation (see above), eluted proteins were separated on SDS-PAGE gels, dried, and subjected to autoradiography. Parallel cultures of cells grown under identical conditions but without the addition of radiolabel were harvested in the presence of phosphatase inhibitors, and proteins were extracted for analysis by immunoblotting with anti-Hac1p.

RESULTS

Hac1p Is Degraded by the Proteasome

In eukaryotic cells, most rapidly turned over proteins are degraded via the ubiquitin–proteasome pathway (Varshavsky, 1997; Hershko and Ciechanover, 1998; Ciechanover *et al*., 2000). We tested the stability of Hac1p in an *S. cerevisiae* strain carrying a temperature-sensitive mutation in the *CIM3* (*SUG1/RPT6*) gene, which encodes an "AAA" ATPase of the 19S regulatory subunit of the proteasome (Ghislain *et al*., 1993). A protein synthesis shutoff assay in which the decay of Hac1p is measured after treatment of cells with cycloheximide was used to compare the half-life of the protein in *cim3–1^{ts}* cells with that in parental W303 cells. Because Hac1p is synthesized only in cells undergoing ER stress, cells grown to OD_{600} of ~0.6 at the permissive temperature of 23°C were first treated with tunicamycin to induce the UPR as the result of accumulation of abnormally glycosylated and malfolded secretory precursors in the ER (Elbein, 1987; Kozutsumi *et al*., 1988). After 90 min, the cultures were shifted to the nonpermissive temperature of 37°C, and 30 min later the cycloheximide shutoff assay was performed as described in *Materials and Methods*. As shown in Figure 1, A and B, the half-life of Hac1p in the parental W303 cells was 1–1.5 min, consistent with the values of 1–2 min obtained previously with other yeast strains by using either protein shutoff or pulse-chase techniques (Chapman and Walter, 1997; Kawahara *et al*., 1997). By contrast, in *cim3–1^{ts}* cells the rate of degradation of Hac1p was significantly decreased, indicating the involvement of the proteasome in the rapid turnover of Hac1p.

Substrate proteins are marked for recognition and degradation by the 26S proteasome by attachment of monoubiquitin to a lysine residue followed by assembly of a tetraubiquitin chain via linkage through lysine 48 of ubiquitin (Thrower *et al*., 2000). To investigate the role of the 11 lysines in Hac1p in ubiquitin-mediated degradation, we analyzed the stability and capacity to confer ER stress resistance of Hac1p mutants in which one or more of the lysine residues were substituted by arginine. These experiments are described in full in the Supplemental Material. The data presented in Supplemental Figure S1 show that none of the mutations significantly affected the stability of the protein, suggesting that as previously observed for other target proteins such as Gcn4p (Kornitzer *et al*., 1994) and Sic1p (Petroski and Deshaies, 2003), no single lysine residue is the sole required attachment point for ubiquitylation-mediating degradation.

Hac1p Harbors a PEST Degron

Many proteins that undergo rapid turnover contain sequence identifiers that target the protein for degradation (Laney and Hochstrasser, 1999). Inspection of the Hac1p sequence failed to identify any common destruction motifs such as the D-box and KEN-box found in cyclins and other cell cycle-regulated proteins (Yamano *et al*., 1998; Vodermaier, 2004). The PESTfind algorithm (Rogers *et al*., 1986) was then used to analyze the Hac1p sequence for potential PEST motifs, which are hydrophilic, enriched in proline, glutamate, serine, and threonine, and at least 12 amino acids in length (Rogers *et al*., 1986; Rechsteiner and Rogers, 1996). The PESTfind program identified one high-confidence PEST sequence (score +6.81) located between residues 125 and 147 of Hac1p (Figure 2A), whose existence had previously been noted by Cox and Walter (1996), and one low-confidence sequence (residues 167–217, score −0.08; location boxed in Figure 2A). Oligonucleotide-directed mutagenesis was used to delete each potential PEST sequence from the *HAC1* coding sequence. Restriction fragments encompassing each deletion were inserted into the *CEN*-based pHAC1 expression vector, replacing the corresponding wild-type sequences. Vectors encoding the wild-type or PEST1- or PEST2-deleted Hac1 proteins were transfected into KMY1045 (Δ*hac1*) cells, and the resulting transformants were grown at 30°C to OD_{600} of 0.6 and then treated with tunicamycin to induce the synthesis of Hac1p before the cycloheximide shutoff assay was performed to compare the rates of turnover of the wild-type and mutant Hac1 proteins. As shown in Figure 2, B and C, the half-life of exogenously expressed wild-type Hac1p in KMY1045 cells (~1.2 min) is essentially identical to that observed for endogenous Hac1p in W303 cells (Figure 1). However, deletion of the PEST1 motif caused a signifi-

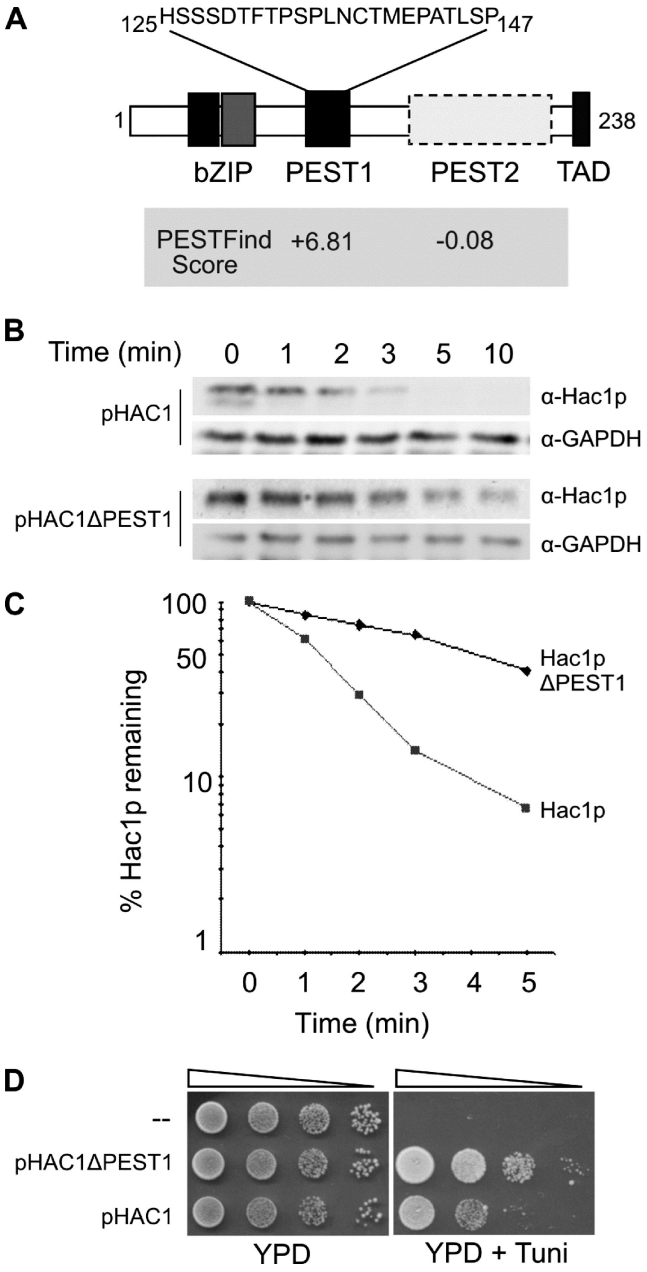

Figure 2. Hac1p stability is dependent on the presence of a functional PEST degron. (A) Schematic diagram indicating regions in the active (238-amino acid) form of Hac1p and showing the scores of potential PEST regions identified using the PESTfind program. (B) Synthesis shutoff assays were performed essentially as described in the legend to Figure 1, except that KMY1045 (Δ*hac1*) cells expressing wild-type or ΔPEST Hac1 proteins were grown at and the assay was performed at 30°C. (C) Plots showing the amount of Hac1p remaining compared with that present at the zero time point before the addition of cycloheximide were generated from the data shown in B by using ImageQuant 4.5 software. (D) Serial 10-fold dilutions of KMY1045 (Δ*hac1*) cells transformed with pHAC1 or pHAC1ΔPEST were spotted on YPD media alone or YPD media containing 1.0 μg/ml tunicamycin (to test for sensitivity to ER stress).

cant increase in the half-life of the protein (to ~4 min), indicating that a degradation signal (degron) located within residues 125-147 of Hac1p plays an important role in the rapid turnover of the protein. By contrast, deletion of the low-scoring PEST2 sequence had no significant effect on the half-life of Hac1p (data not shown). Consistent with its greater stability, Δ*hac1* cells expressing the PEST1-deleted Hac1p protein from the pHAC1ΔPEST1 vector were more resistant to ER stress than were those expressing wild-type Hac1p from pHAC1 (Figure 2D).

To determine whether the Hac1p PEST1 (hereinafter called PEST) degron is able to target a reporter protein for rapid degradation, we constructed a vector capable of expressing an HA-tagged Ura3 protein, and then inserted *HAC1* nucleotides encoding a sequence spanning the PEST degron (residues 122-158) in frame between the ATG initiation codon and the sequences encoding Ura3/HAp (Figure 3A). W303 (*trp1 ura3*) cells expressing the Ura3/HA protein were equally viable in synthetic media containing or lacking uracil (Figure 3B), demonstrating that the presence of the HA tag at the C terminus of Ura3p had no significant effect on the ability of the enzyme to support uracil biosynthesis. By contrast, cells expressing the PEST-Ura3/HA protein were unable to grow in medium lacking uracil (Figure 3B). Synthesis shutoff assays (Figure 3C) showed that the Ura3/HA fusion protein has a half-life of ~60 min (Figure 3C, top). Consistent with its failure to support cell viability in the absence of uracil, the PEST-Ura3/HA fusion protein was much less stable, being almost completely degraded by the 30-min time point (Figure 3C, bottom). Interestingly, the PEST–Ura3/HA fusion protein, unlike the Ura3/HA protein, was very significantly stabilized at the nonpermissive temperature in MT670 cells, which have a temperature-sensitive defect in the Ubc3p/Cdc34p E2 ubiquitin-conjugating enzyme (Figure 3D). This is the same E2 enzyme that we show below to mediate degradation of Hac1p. Because other degradation signals target a similar Ura3/HA fusion protein to different E2 enzymes such as Ubc6p and/or Ubc7p (Gilon *et al*., 1998), our data suggest that residues 122–158 of Hac1p contain a specific recognition signal for Ubc3p/Cdc34p.

Degradation of Hac1p Involves the E2 Ubiquitin-conjugating Enzyme Ubc3/Cdc34p

Ubiquitylation of proteins for targeting to the proteasome requires a multienzyme system involving E1, E2, and E3 enzymes, respectively, for the activation, conjugation and ligation of ubiquitin (for review, see Ciechanover *et al*., 2000; Pickart and Eddins, 2004). In *S. cerevisiae*, a single gene encodes the E1 ubiquitin-activating enzyme. However, 11 different genes encode E2 ubiquitin-conjugating enzymes (Pickart and Eddins, 2004). To identify which E2 enzyme(s) is involved in the turnover of Hac1p, we first tested the stability of the protein in a set of deletion strains that variously lack one (*UBC1*, *UBC2*, *UBC4*, *UBC5*, *UBC7*, *UBC8*) or two (*UBC1* and *UBC4*, *UBC2* and *UBC4*, *UBC6* and *UBC7*) of the nonessential E2 genes that have been demonstrated to play a role in ubiquitin-mediated degradation (Chen *et al*., 1993). No significant differences were observed in the rate of turnover of Hac1p in these strains compared with the parental W303 strain (data not shown). We then analyzed the stability of Hac1p in MT670 cells, which as noted above have a temperature-sensitive defect in the essential *UBC3/CDC34* gene. As shown in Figure 4A, and as anticipated from our data with the PEST–Ura3/HA fusion protein (Figure 3D), the $cdc34^{ts}$ defect caused significant stabilization of Hac1p.

Hac1p Is Stabilized in Yeast Mutants Defective in Components of the SCF^{Cdc4} E3 Complex

E3 enzymes or enzyme-complexes bind E2 enzymes and provide the specificity of ubiquitin-dependent proteolysis by recognizing particular substrates through dedicated interaction domains (for review, see Ciechanover *et al*., 2000;

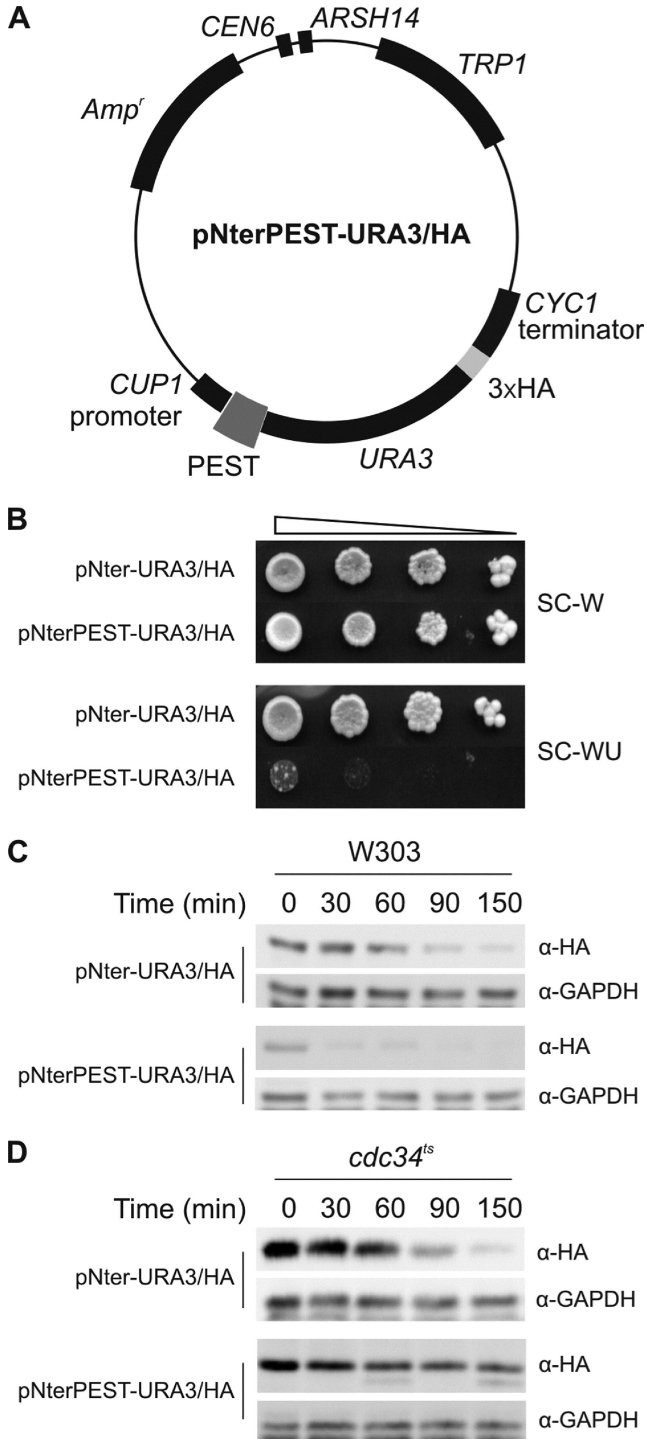

Figure 3. Hac1p PEST degron confers proteolytic instability on Ura3p. (A) The pNterPEST-URA3/HA plasmid was generated as described in the Supplemental Material to check the degron activity of the Hac1p PEST sequence. (B) Serial 10-fold dilutions of W303 cells transformed with pNter-URA3/HA (empty vector) or pNterPEST-URA3/HA were plated on SC media lacking tryptophan (to select for retention of the plasmid) or SC media lacking both tryptophan and uracil (to test for cell viability in the absence of uracil). (C) Synthesis shutoff assays were performed as described in the legend to Figure 2B but over the time course 0–150 min on W303 cells transformed with pNter-URA3/HA or pNterPEST-URA3/HA. (D) Synthesis shutoff assays were performed as described in C but on MT670 (*cdc34^{ts}*) cells transformed with pNter-URA3/HA or pNterPEST-URA3/HA.

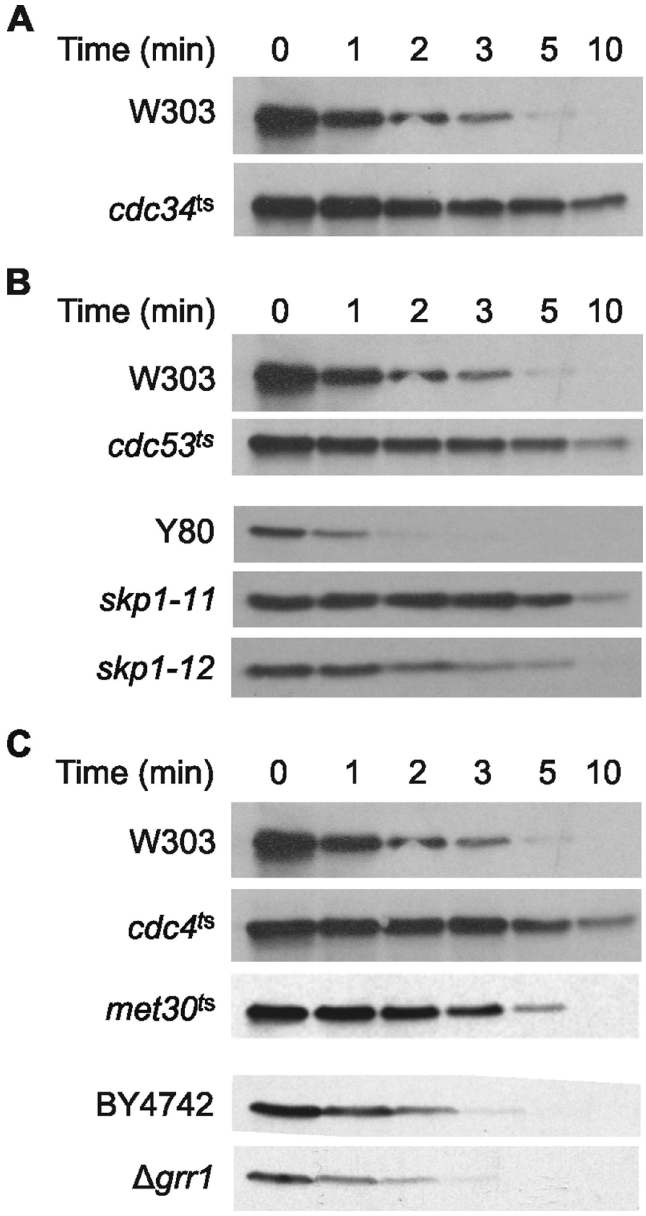

Figure 4. Proteolytic turnover mediated by the Hac1p degron requires components of the SCF^{Cdc4} pathway. (A) Hac1p protein is stabilized in MT670 cells temperature sensitive for the E2 ubiquitin-conjugating enzyme Ubc3 (Cdc34p). Synthesis shutoff assays on MT670 and parental W303 cells were performed as described in the legend to Figure 1A. (B) The Cdc53 cullin and Skp1 core components of the SCF^{Cdc4} E3 ubiquitin ligase complex are required for rapid turnover of Hac1p. Synthesis shutoff assays on MT871 (*cdc53^{ts}*), Y552 (*skp1-11^{ts}*), and Y554 (*skp1-12^{ts}*) cells and their respective parental W303 and Y80 strains were performed as described in the legend to Figure 1A. (C) The Cdc4 F-box protein is required for rapid turnover of Hac1p. Synthesis shutoff assays on MT668 (*cdc4^{ts}*) and PY283 (*met30^{ts}*) and their respective parental W303 and PY1 strains were performed as described in the legend to Figure 1A. Assays on deletion strain *grr1*Δ and its BY4742 parent strain were performed as described in the legend to Figure 2B.

Weissman, 2001; Pickart and Eddins, 2004; Cardozo and Pagano, 2004). In *S. cerevisiae*, the Ubc3/Cdc34 E2 enzyme functions in conjunction with SCF E3 complexes, which contain the RING domain protein Rbx1p in addition to the

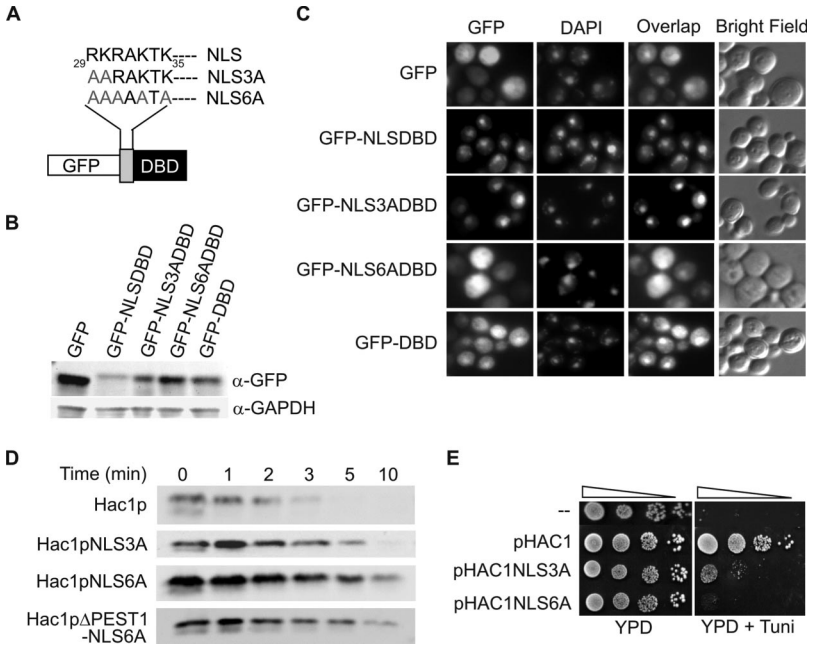

Figure 5. Hac1p degradation correlates with nuclear localization. (A) Wild-type and mutant versions of an NLS present near the N terminus of Hac1p. (B) Extracts of W303 cells expressing unfused GFP (empty vector) or fusion proteins containing wild-type or mutant versions of the Hac1 NLSDBD sequence were analyzed by immunoblotting using α-GFP and α-GAPDH antibodies. (C) W303 cells expressing unfused GFP (empty vector) or fusion proteins containing wild-type or mutant versions of the Hac1 NLSDBD sequence were analyzed by fluorescence microscopy. (D) Synthesis shutoff assays were performed as described in Figure 2A on KMY1045 (*hac1*Δ) cells transformed with pHAC1, pHAC1NLS3A, pHAC1NLS6A, pHAC1ΔPEST, and pHAC1NLS6AΔPEST. (E) Serial 10-fold dilutions of KMY1045 (Δ*hac1*) cells transformed with pHAC1, pHAC1NLS3A, and pHAC1NLS6A were spotted on YPD media alone or YPD media containing 0.5 μg/ml tunicamycin (to test for sensitivity to ER stress).

Cdc53/Cul1 scaffold protein, the Skp1 adaptor protein, and an F-box protein that recruits substrates via a specific protein–protein interaction domain, such as a WD40 or leucine-rich domain (for review, see Deshaies, 1999; Willems *et al*., 2004). We confirmed the involvement of an SCF complex in the degradation of Hac1p by measuring the stability of endogenous Hac1p in *S. cerevisiae* cells bearing temperature-sensitive defects in the Cdc53 or Skp1 proteins, which are core components of yeast SCF complexes (Deshaies, 1999; Xu *et al*., 2003; Willems *et al*., 2004; Vodermaier, 2004). As shown in Figure 4B, the half-life of Hac1p was increased in *cdc53*^{ts} and *skp1-11*^{ts} cells compared with that in the parental cells to an extent very similar to that observed for the *cdc34*^{ts} mutation. *skp1-12*^{ts} cells also displayed slower turnover of Hac1p, but the effect was less than that observed with the *skp1-11*^{ts} allele.

S. cerevisiae cells contain at least 21 F-box–containing proteins (Willems *et al*., 2004). Of these, four (Cdc4p, Grr1p, Met30p, and Dsg1/Mdm30p) are components of SCF complexes required for degradation of various cell cycle regulatory proteins and transcription factors (for review, see Deshaies, 1999; Willems *et al*., 2004; Muratani *et al*., 2005). Proteomic approaches showed that a further three (Ydr131c, Yjl149w, and Ylr097c) form complexes with Cdc53p (Willems *et al*., 1999). To identify the F-box protein(s) required for rapid degradation of Hac1p, we analyzed the stability of Hac1p in cells having temperature-sensitive mutations in the *CDC4* or *MET30* genes, or lacking the *GRR1*, *DSG1*, *Ydr131c*, *Yjl149w*, or *HRT3* genes, or lacking both the *MET30* and *MET4* genes. We also analyzed Hac1p stability in an additional 11 deletion strains (Table 1) lacking genes encoding F-box motif-containing proteins that have not yet been shown to form SCF complexes. Of the 19 mutants examined, only strain MT668 (temperature sensitive for Cdc4p) displayed any difference in the stability of Hac1p compared with its parent cell line (Figure 4C; data not shown). At the nonpermissive temperature, the half-life of Hac1p in *cdc4*^{ts} cells was increased to ~5 min, a value similar to that observed with the *cdc34*^{ts}, *cdc53*^{ts}, and *skp1-11*^{ts} mutants. We therefore concluded that rapid degradation of Hac1p involves the SCF^{Cdc4} E3 complex.

Degradation of Hac1p Correlates with its Nuclear Localization

Consistent with the finding that the cellular localization of Cdc4p is exclusively nuclear (Blondel *et al*., 2000), the degradation of the SCF^{Cdc4}-targeted substrates Far1p and Gcn4p requires their presence in the nucleus (Blondel *et al*., 2000; Pries *et al*., 2002). To determine whether rapid turnover of Hac1p also requires its nuclear localization, we first examined the function of a potential nuclear localization sequence (NLS) located at or near the N-terminal end of the bZIP domain of the protein (Figure 5A). This sequence, $_{29}$RKRAKTK$_{35}$, contains a cNLS consensus motif (KR/KxR/K; Fontes *et al*., 2000). Site-directed mutagenesis using the QuikChange PCR method (Wang and Malcolm, 1999) was used to substitute alanine residues for either the first two or all five basic residues of the NLS to generate the NLS3A and NLS6A mutations (Figure 5A). A ΔNLS mutation that removed all seven residues of the putative cNLS also was constructed. The mutated sequences were incorporated either into the pGFP-NLSDBD vector that encodes a GFP fusion protein containing the NLS and DNA binding domain (DBD) sequences (amino acids 29–65) of Hac1p, or into the pHAC1 or pHAC1ΔPEST vectors that encode the full-length or ΔPEST Hac1 proteins (see *Materials and Methods*). The DBD sequences were included in the GFP fusion proteins to test the possibility that additional basic sequences within the DNA binding domain contribute to nuclear import.

The parental pGFP-Nfus plasmid and the pGFP-NLSDBD vectors were introduced into W303 yeast cells, and the expression levels of GFP and the various fusion proteins were analyzed by immunoblotting with an anti-GFP antibody (Figure 5B), whereas the intracellular localization of GFP was analyzed by confocal microscopy (Figure 5C). As shown in the left-hand panel of the top row of Figure 5C, cells expressing unfused GFP displayed both cytoplasmic and nuclear fluorescence, with the exception of a dark patch that does not colocalize with the nucleus (shown by DAPI staining in the middle panels), but probably corresponds to the vacuole. When the wild-type NLSDBD sequence was attached to GFP, the fusion protein was efficiently targeted to the nucleus (Figure 5C, second row), confirming that this

sequence does indeed contain a functional NLS. In comparison, cells expressing the GFP–NLS3ADBD fusion protein displayed a relatively small increase in cytoplasmic fluorescence (Figure 5C, third row), whereas those expressing the GFP–NLS6ADBD fusion protein displayed a greatly reduced proportion of fluorescence in the nucleus. The residual nuclear fluorescence occurred at a level only slightly higher than that in the surrounding cytoplasm and very similar to that exhibited in cells expressing either unfused GFP (Figure 5C, top row) or the GFP–DBD fusion protein (Figure 5C, bottom row). We therefore concluded that substitution by alanine of the first two basic residues of the RKRAKTK sequence only slightly reduced NLS activity, whereas substitution of all the basic residues in this sequence, or deletion of the whole sequence, completely disrupted its ability to promote nuclear localization of GFP. The basic residues in the remainder of the DBD sequence do not seem to contribute any NLS activity.

We then tested the stability of full-length Hac1 proteins containing the same alanine substitution mutations using synthesis shutoff assays (Figure 5D), which demonstrated that the NLS3A and NLS6A mutants have significantly longer half-lives than the wild-type Hac1 protein, with that of the NLS6A mutant approaching that of the ΔPEST mutant. The degree of stabilization of the NLS mutants correlated with the severity of the nuclear localization defect, supporting the hypothesis that localization of Hac1p in the nucleus is necessary for its SCF^{Cdc4}-dependent turnover. The combination of the ΔPEST and NLS6A mutations did not further increase the stability of Hac1p above that seen for the NLS6A mutation alone, consistent with the majority of the protein being localized in the cytoplasm where it would not be available for SCF^{Cdc4}-dependent degradation. Finally, we tested the capacity of Hac1 proteins containing the alanine substitution mutations to support the viability of cells under ER stress conditions. Expression of wild-type Hac1p from the pHAC1 vector fully complemented the sensitivity of KMY1045 (Δ*hac1*) cells to the presence of 0.5 μg/ml tunicamycin (compare top two rows of Figure 5E). However, cells expressing the NLS3A and NLS6A mutant proteins displayed considerably reduced viability under ER stress conditions, with the degree of sensitivity to tunicamycin correlating inversely with both the extent of nuclear localization and the stability of the mutant proteins.

The PEST-dependent Interaction of Hac1p with Cdc4p Requires Ser146 and Ser149

To confirm that Hac1p forms a complex with Cdc4p, we fused the N-terminal 195 amino acids of Hac1p (Figure 6A), which include the PEST degron but not the transactivation domain (residues 221–238; Mori *et al*., 2000), to the LexA DNA binding domain and compared its ability to interact in a two-hybrid experiment with a fragment of Cdc4p (residues 339-779) that contains WD40 repeats (Fong *et al*., 1986; Drury *et al*., 1997). A known binding partner, the N-terminal 47 amino acids of Cdc6p (Drury *et al*., 1997; Perkins *et al*., 2001), was used as a positive control. The data shown in Figure 6B (rows 1 and 2) demonstrate that the N-terminal 195 residues of Hac1p, like the N-terminal 47 amino acids of Cdc6p, interacted strongly with the WD40 repeat-containing fragment of Cdc4p, indicating that this region of Hac1p can act as a Cdc4-interaction domain in vivo. Deletion of the Hac1 PEST degron abrogated the interaction (Figure 6B, row 3), supporting our earlier conclusion that the PEST sequence is necessary for Cdc4p-mediated degradation of Hac1p.

All known substrates of SCF^{Cdc4} must be phosphorylated before they can be ubiquinated (for review, see Deshaies,

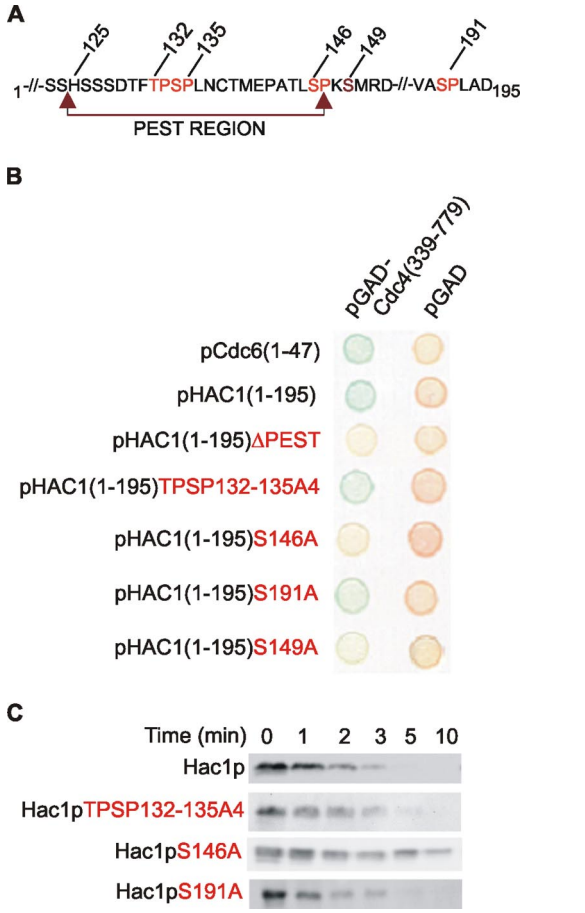

Figure 6. Interaction between Hac1p and Cdc4p is dependent on the presence of the Hac1 PEST sequence, and, in particular, Ser146 and Ser149. (A) Partial polypeptide sequence of Hac1p showing the PEST degron and putative Cdc4 binding sites. (B) W303 cells cotransformed with plasmids pGAD or pGAD-Cdc4(339-779) and plasmids containing *LexA* (DBD) fusions were assayed for β-galactosidase activity as indicated by development of blue coloration. The column on the left shows cells expressing the GAD–Cdc4(339-779) fusion protein. The column on the right shows cells expressing GAD alone. Cells also expressed *LexA* DBD fused to various mutant forms of Hac1p (residues 1-195) or Cdc6p (residues 1-47) (as a positive control). (C) Synthesis shutoff assays were performed as described in Figure 2A on KMY1045 (Δ*hac1*) cells transformed with pHAC1 or pHAC1-based vectors encoding the indicated Hac1p mutations.

1997; Willems *et al*., 1999, 2004), and the WD40 repeat domain of Cdc4 binds with high-affinity to a consensus phosphopeptide motif I/L-I/L/P-pT-P-{K/R}$_4$ (where {} indicates disfavored residues) called the *C*dc4 *P*hospho-*D*egron or CPD (Nash *et al*., 2001). It is noteworthy that the CPD motif contains the minimal consensus sequence S/T-P for phosphorylation by both cyclin-dependent kinases (CDKs) and mitogen-activated protein (MAP) kinases (Nigg, 1995), and indeed members of these kinase families are required for SCF^{Cdc4}-dependent degradation of several different substrates (for review, see Willems *et al*., 2004). Examination of the sequence of Hac1p revealed four S/T-P motifs, of which three ($T_{132}P$, $S_{134}P$, and $S_{146}P$) lie within the PEST degron and are each embedded in the sequence context of a putative CPD (Figure 3A). Substitution by alanines of residues 132–135, which removes the first two motifs, had no effect on the capacity of LexA-Hac1(1-195) to interact with GAD-

Cdc4(339-779) in the two-hybrid assay (Figure 6B, row 4). However, substitution of Ser146 by alanine completely inhibited the interaction (Figure 6B, row 5), indicating that the sequence TLS_{146}PKSMR is a functional CPD, despite the presence of two "disfavored" basic residues in the +2 and +5 positions of the motif. It is noteworthy that this CPD does not lie entirely within the PEST1 sequence defined by the PESTfind algorithm (Figure 2A), but the deletion of residues 125–147 that proved this sequence to contain a functional degron (Figure 2B) removed five of the eight residues of the CPD, including the S_{146}-P phosphorylation site. Importantly, the complete CPD was included in the slightly longer sequence (residues 122–158) that promoted the degradation of the PEST–Ura3/HA fusion protein (Figure 3C). Consistent with Ser191 not lying within a CPD motif, its substitution by alanine had no effect on the Hac1–Cdc4 interaction (Figure 6B, row 6). When the half-lives of full-length Hac1 proteins containing the same alanine substitution mutations were measured using the synthesis shutoff assay and compared with those of the wild-type protein and the PEST deletion mutant, we found that their stabilities showed an inverse correlation with their capacities to interact with Cdc4p (Figure 6C). Thus, the instability of the TPSP132-135A and S191A mutants resembled that of wild-type Hac1p, whereas the stability of the S146A mutant was increased to an extent very similar to that of the PEST deletion mutant, confirming the role of the CPD in rapid turnover of Hac1p and the importance of Ser146 in this process.

Included within the Hac1 CPD is a consensus protein kinase C (PKC) phosphorylation site, S_{149}MR, which had come to our attention previously because osmotic shock conditions that activate Pkc1p, the sole member of the PKC family in *S. cerevisiae* (Levin *et al*., 1990), cause an increase in the steady-state level of Hac1p (see below). Substitution of Ser149 by alanine increased the half-life of Hac1p to ~5 min (Dyke, 2003). Introduction of the S149A mutation into the LexA–Hac1(1-195) fusion protein caused a very significant but not complete inhibition of its interaction with GAD-Cdc4 (Figure 6B, row 7), indicating that phosphorylation of S149 may play an important but not absolutely essential role in the recognition of the CPD by Cdc4p.

The S146A Mutation in the SCF^{Cdc4} Binding Site Results in Increased Cell Viability and UPRE-dependent Transcription under ER Stress Conditions

Two assays demonstrated the effect of the S146A mutation in improving the function of Hac1p in vivo. First, KMY1045 (Δ*hac1*) cells transformed with a single-copy vector encoding the Hac1pS146A mutant were ~10-fold less sensitive to ER stress caused by growth in the presence of 1 μg/ml tunicamycin than cells transformed with the same vector encoding wild-type Hac1p (Figure 7A). The magnitude of this increased resistance to ER stress is very similar to that observed with the ΔPEST mutation (Figure 2D). Second, the S146A mutant displayed 1.8- to 2-fold higher capacity than the wild-type protein in inducing the expression of β-galactosidase from the UPRE-*CYC1-lacZ* reporter construct (Figure 7B). This relatively modest increase is consistent with the finding that the *KAR2* gene, the origin of the 22 base pairs UPRE used in the UPRE-*CYC1-lacZ* reporter construct, is a member of a class of UPR-target genes whose transcription does not increase linearly in proportion to the concentration of Hac1p (Leber *et al*., 2004). This is probably because Hac1p binds to the UPRE sequences of these genes with high affinity, so that binding is at or near saturation level at the concentration of Hac1p that accumulates under normal ER stress conditions. Other classes of UPR target genes require higher concentrations of Hac1p to achieve maximal levels of transcription of their target proteins (Leber *et al*., 2004). The 10-fold increase in ER stress resistance observed in the experiments shown in Figures 2D and 7A probably reflects the effect of stabilization of Hac1p by the ΔPEST and S146A mutations on the transcription of target genes belonging to these latter classes.

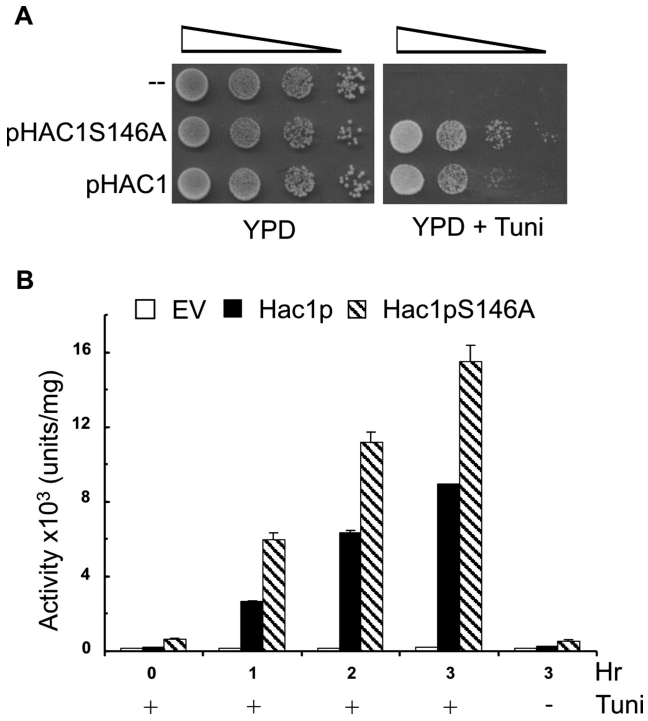

Figure 7. S146A mutation in the SCF^{Cdc4} binding site results in increased cell viability and UPRE-dependent transcription under ER stress conditions. (A) Serial 10-fold dilutions of Δ*hac1* cells transformed with pHAC1 or pHAC1S146A were spotted on YPD media alone or YPD media containing 1.0 μg/ml tunicamycin (to test for sensitivity to ER stress). (B) NCY1810 (Δ*hac1*) cells cotransformed with the pMCZY multicopy UPRE-*CYC1-LacZ* reporter construct (Mori *et al*., 1993) and the pRS315 empty vector or pHAC1-based plasmids encoding wild-type Hac1p or the S146A Hac1p mutant were grown overnight at 30°C in SC-Leu/Ura–selective media until the OD_{600} reached 0.8. Incubation of the cultures was then continued in the presence (+) or absence (−) of 5 μg/ml tunicamycin for the time intervals (0–3 h) indicated. Protein extracts were prepared, and assays of β-galactosidase activity were carried out as described in *Materials and Methods*. Comparisons of the transcriptional activities (units per milligram of total cell protein) of the three strains were performed in triplicate with at least two independent transformants. The results are presented as mean ± SD.

Srb10 Kinase Activity Is Essential for Efficient Degradation of Hac1p

In yeast, Cdc4p-dependent degradation of CDK inhibitors like Sic1p and Far1p and the DNA replication regulator Cdc6p require the activity of the cell cycle CDK Cdc28p (Henchoz *et al*., 1997: Verma *et al*., 1997; Elsasser *et al*., 1999). In contrast, degradation of the transcriptional activator Gcn4 involves its phosphorylation by Srb10 or Pho85 kinases (Meimoun *et al*., 2000; Chi *et al*., 2001). We used a Δ*srb10* strain to test whether the Srb10 kinase, which together with its activator the Srb11 cyclin is a component of the SRB mediator complex (Myer and Young, 1998), is required for degradation of Hac1p. As shown in Figure 8A, synthesis

shutoff assays demonstrated that the half-life of Hac1p was significantly increased in JN5 (Δ*srb10*) cells compared with that in parental JN1 cells. Δ*srb10* cells were also significantly (~50-fold) less sensitive to ER stress than the parental cells (Figure 8B). To determine whether effects of the S146A mutation and the absence of the Srb10 kinase are additive, we deleted the *SRB10* gene from KMY1045 (Δ*hac1*) cells to generate the NCY1811 (Δ*hac1*Δ*srb10*) strain and then compared the stability of exogenous wild-type and S146A Hac1 proteins in the Δ*hac1* and Δ*hac1*Δ*srb10* cells. Consistent with the earlier experiments (Figures 6C and 8A), each of the single S146A or Δ*srb10* mutations increased the stability of the Hac1 protein (Figure 8C, compare the first three sets of panels). The combination of the two mutations resulted in even greater stability (Figure 8C, bottom), indicating that Srb10 kinase-mediated phosphorylation of S146 is not the sole pathway of activation of turnover of Hac1p. This additive effect of the S146A and Δ*srb10* mutations on the stability of Hac1p was reflected in Δ*hac1*Δ*srb10* cells expressing Hac1pS146A being significantly less sensitive to ER stress than those expressing wild-type Hac1p (Figure 8D).

Activation of the Cell Integrity MAP Kinase Pathway Stabilizes Hac1p

In a separate study we recently showed that overexpression of components of the cell integrity (CI) MAP kinase signal transduction pathway, which regulates cell integrity by upregulating cell wall synthesis in response to weakening or stretching of the cell wall (Gustin *et al.*, 1998; Heinisch *et al.*, 1999), suppresses the transcriptional defect of a reporter gene controlled by a point mutated UPRE element (Helfenbaum, unpublished data). This raised the possibility of a previously unrecognized interaction between the UPR and CI responses. To investigate how activation of the CI pathway by hypotonic shock affects the UPR, KMY2105 *sec53^{ts}* cells containing an integrated UPRE-*lacZ* reporter gene were grown to mid-logarithmic phase in medium containing 1 M sorbitol at the permissive temperature of 23°C. *sec53^{ts}* cells are defective in phosphomannomutase (Kepes and Schekman, 1988) and activate the UPR at the semipermissive temperature of 30°C due to the accumulation of abnormally glycosylated and malfolded secretory precursors in the ER (Normington *et al.*, 1989). The cells were then treated with osmotic diluents as described in the legend to Figure 9A and simultaneously subjected to UPR stress by raising the incubation temperature to 30°C. Control cultures were treated with osmotic diluents but were incubated at 23°C. At various times (0–3 h) after dilution, aliquots of the culture were collected for assay of β-galactosidase activity. Figure 9A shows that the UPR, measured by expression of β-galactosidase from the reporter gene, was accelerated when cells were transferred into hypotonic conditions at the same time as UPR stress was applied. Hypotonic shock does not itself activate the UPR in the absence of ER stress, because no induction of β-galactosidase activity was observed if the cells were maintained at 23°C throughout the experiment. Essentially identical effects of hypotonic shock were obtained using *SEC^{+}* cells grown at 30°C with the UPR being induced using tunicamycin in the absence of any temperature shift (data not shown).

To determine whether the time course of UPR-induced accumulation of Hac1p was similarly affected by osmotic conditions, cell extracts collected in an experiment similar to that shown in Figure 9A were immunoblotted with an antibody specific for the C terminus of Hac1p, which is encoded by the *HAC1* 3′ exon and translated only from spliced *HAC1* mRNA (Cox and Walter, 1996). Figure 9B shows that Hac1p accumulated faster and to higher levels in hypotonic

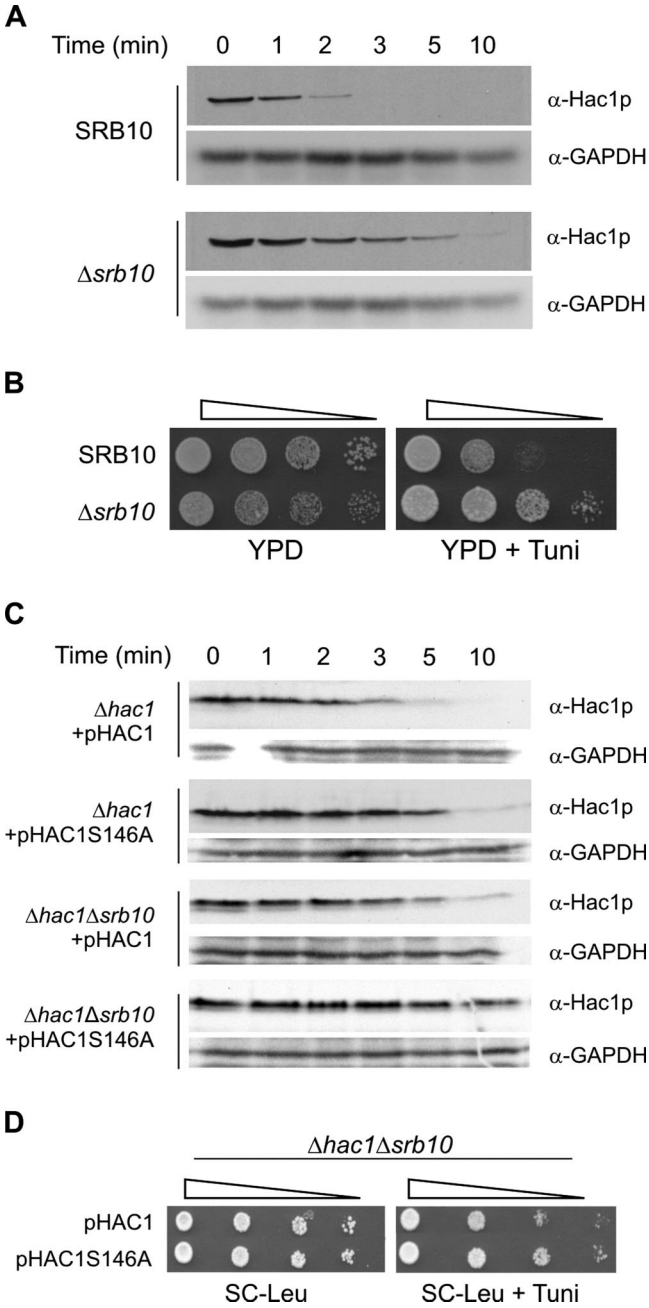

Figure 8. Srb10 kinase activity is essential for efficient degradation of Hac1p. (A) Synthesis shutoff assays were performed as described in the legend to Figure 1A on JN5 (Δ*srb10*) and parental JN1 cells. (B) Serial 10-fold dilutions of Δ*srb10* and parental cells were spotted on YPD media alone or YPD media containing 1.0 μg/ml tunicamycin to test for sensitivity to ER stress. (C) Synthesis shutoff assays were performed as described in the legend to Figure 2B on NCY1811 (Δ*hac1* Δ*srb10*) and parental KMY1045 (Δ*hac1*) cells transformed with pHAC1 or pHAC1S146A. (D) Serial 10-fold dilutions of Δ*hac1* Δ*srb10* cells transformed with pHAC1 or pHAC1S146A were spotted on SC-Leu media alone or SC-Leu media containing 1.0 μg/ml tunicamycin.

shock conditions compared with isotonic conditions. To investigate to what extent stabilization of Hac1p might contribute to this increased accumulation of Hac1p, we then used synthesis shutoff assays to analyze the effect of hypotonic shock on the half-life of the protein. As shown in

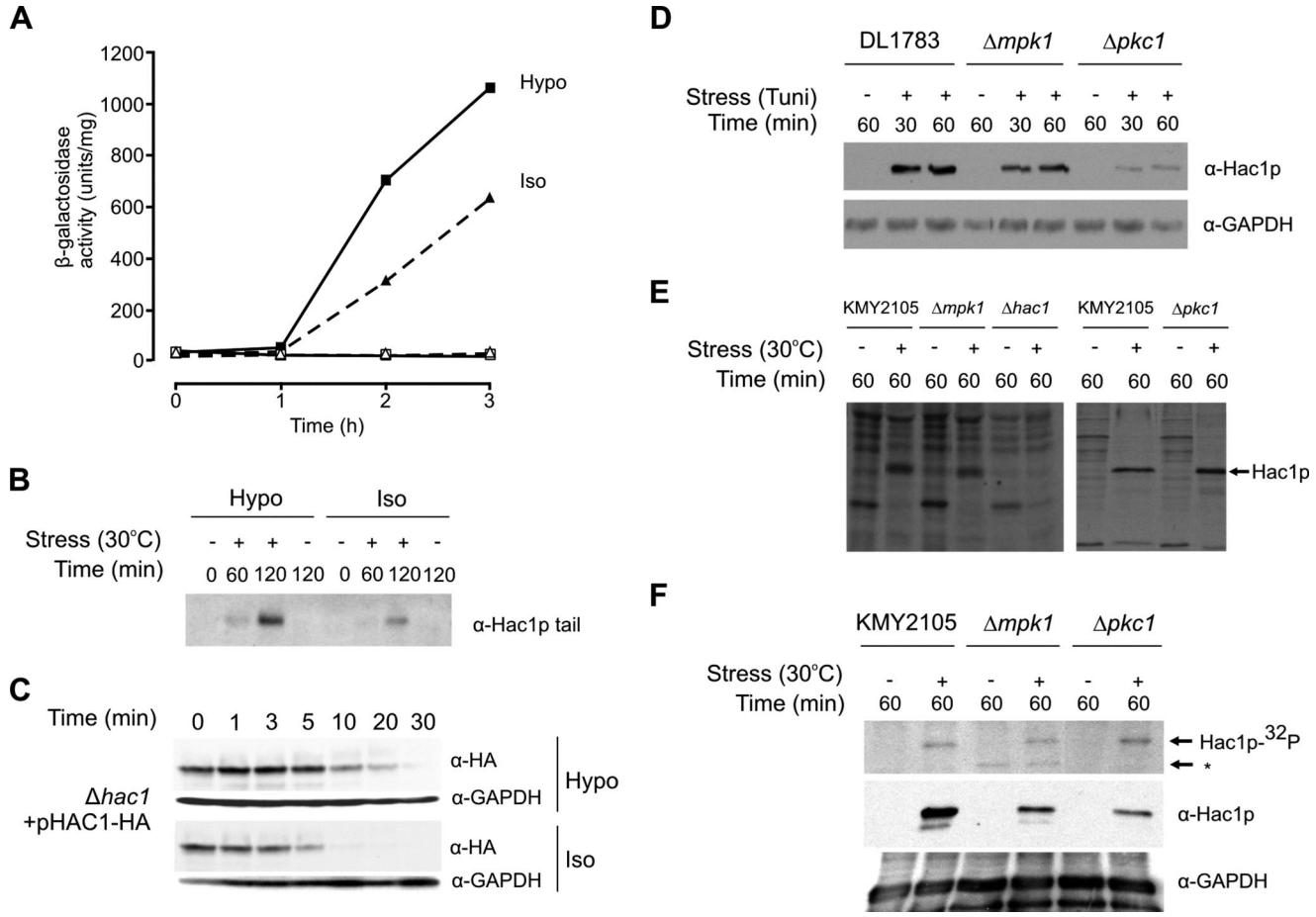

Figure 9. Modulation of the UPR by osmotic shock involves stabilization of Hac1p. (A) KMY2105 cells (*sec53*^{ts} cells containing a chromosomally integrated UPRE-*lacZ* reporter; Kawahara *et al*., 1997) were grown to mid-logarithmic phase at 23°C in 20% YPD medium supplemented with 1 M sorbitol. Aliquots of the culture were then subjected to UPR stress under isotonic (triangles) or hypotonic (squares) shock conditions by dilution with 4 parts of prewarmed (30°C, closed symbols) osmotic diluents (0.4% glucose for hypotonic, 0.4% glucose + 1 M sorbitol for isotonic). Zero-time samples were removed to ice immediately and incubation was continued for 3 h at 30°C. Control cultures (open symbols) were treated with osmotic diluents but were incubated at 23°C. At various times (0–3 h) after dilution, aliquots of the cultures were collected for assay of β-galactosidase activity as described in *Materials and Methods*. The results (units per milligram of total cell protein) are presented as mean ± SD based on duplicate determinations in two separate experiments. (B) KMY2105 cells transformed with pHAC1B were grown in SC-Leu supplemented with 1 M sorbitol. The cells were treated as described for A and then incubated for 0–120 min before aliquots were collected and the cells snap-frozen in liquid nitrogen before extracts were prepared essentially as described by Cox *et al*. (1993). Hac1p protein was detected by immunoblotting using anti-Hac1p-tail antibody. (C) KMY1045 cells transformed with pHAC1-HA were grown to OD_{600} of 0.6 in SC-Leu supplemented with 1 M sorbitol. The culture was then subjected to UPR stress for 60 min by using dithiothreitol (DTT; 6 mM final concentration) to prevent correct disulfide bond formation. Aliquots of the culture were then subjected to isotonic or hypotonic shock conditions by diluting with 4 volumes of prewarmed (30°C) osmotic diluents containing cycloheximide (SC-Leu media for hypotonic and SC-Leu supplemented with 1 M sorbitol for isotonic; final concentration of cycloheximide 1 mg/ml). Zero-time samples were removed and snap-frozen in liquid nitrogen as described in *Materials and Methods*. Incubation was continued at 30°C with further samples taken at the indicated time points and snap-frozen as described above. Cell extracts were then prepared and analyzed by immunoblotting with anti-HA tag and then anti-GAPDH as described in *Materials and Methods*. (D) DL1783 (parental), DL455 (Δ*mpk1*), and DL376 (Δ*pkc1*) cells were grown to OD_{600} of 0.8 at 30°C in selective medium supplemented with 1 M sorbitol. The cultures were then divided in two and the cells incubated for a further 1 h in the presence (+) or absence (−) of tunicamycin. Aliquots were collected at the indicated times and cell extracts were prepared as described in B before Hac1p proteins were detected by immunoblotting using anti-Hac1p antibody. The filters were then stripped and reprobed using anti-GAPDH antibodies. Then, 100 μg of total cell protein was loaded in each lane. (E) KMY2105, LHY100 (Δ*mpk1*), KMY2145 (Δ*hac1*), and LHY102 (Δ*pkc1*) cells were grown at 23°C in selective medium (left) or in selective medium supplemented with 1 M sorbitol (right). Cells were labeled for 5 min with ^{35}S[methionine and cysteine] after induction of UPR stress (30°C for 60 min) or after incubation at the permissive temperature of 23°C. Hac1p immunoprecipitated from cell lysates was subjected to SDS-PAGE and detected by fluorography. (F) KMY2105, LHY100 (Δ*mpk1*), and LHY102 (Δ*pkc1*) cells grown in selective medium supplemented with 1 M sorbitol were labeled with [^{32}P]orthophosphoric acid as described in *Materials and Methods* before UPR stress was imposed for 60 min by raising the temperature to 30°C. Control cultures were maintained at 23°C. Top, Hac1p was immunoprecipitated from protein extracts prepared as described by Cox and Walter (1996) and separated by SDS-PAGE. The gels were dried and autoradiographed. Hac1p-^{32}P indicates Hac1p identified by autoradiography, whereas the asterisk indicates a nonspecific band not reproducibly observed in other experiments. Middle and bottom, parallel cultures were treated as described above but not radiolabeled. Whole cell lysates (100 μg/lane) were analyzed by immunoblotting, first using anti-Hac1p (middle) and then anti-GAPDH antibodies (bottom).

Figure 9C, the half-life of Hac1p was significantly increased under hypotonic shock conditions.

Interestingly, the sequences surrounding Ser146 and Ser149 in Hac1p make these residues potential sites of phosphorylation by the Pkc1p and/or Mpk1p kinases, which are important components of the CI signaling pathway (Levin *et al*., 1990; Lee and Levin, 1992). However, Pkc1p- or Mpk1p-mediated phosphorylation of these residues should activate (in Ser146) or stimulate (in Ser149) degradation of Hac1p, which is inconsistent both with activation of the CI pathway causing stabilization of Hac1p and with deletion of *PKC1* or *MPK1* causing significantly decreased accumulation of Hac1p after UPR stress, both under normal osmotic conditions (Figure 9D) and after hypotonic shock (Helfenbaum, unpublished data). This decreased accumulation was not due to decreased synthesis of Hac1p in the deletion strains (Figure 9E). Because deletion of either kinase resulted in increased overall phosphorylation of Hac1p (Figure 9F), we think that Pkc1p and or Mpk1p may inhibit or activate, respectively, other kinases or phosphatases that act upon Hac1p.

DISCUSSION

Many transcription factors are among the most rapidly degraded cellular proteins and their high rates of turnover provide tight coupling of their transcriptional activity to their continued synthesis. Previous studies on the regulation of the UPR have concentrated on the initiation of signaling, which results in the splicing of constitutively expressed *HAC1* precursor mRNA by a nonconventional mechanism that releases the blockage of translation of Hac1p (for review, see Kaufman, 1999; Patil and Walter, 2001). Hac1p has been known for some time to be an extremely short-lived transcriptional activator (Chapman and Walter, 1997; Kawahara *et al*., 1997), but to date the sequence elements in Hac1p and the cellular machinery that facilitate its turnover had not been characterized. In this study, we demonstrated that Hac1p harbors a functional PEST sequence (residues 125–147) that is required for its rapid degradation. Furthermore, we showed that proteolysis of Hac1p by the proteasome involves the E2 ubiquitin-conjugating enzyme Ubc3/Cdc34p and the SCF^{Cdc4} E3 complex. Consistent with the known nuclear localization of the F box protein Cdc4p, rapid degradation of Hac1p requires the presence of a nuclear localization sequence, whose function depends upon basic residues in the sequence $_{29}$RKRAKTK$_{35}$. Two-hybrid analysis demonstrated that the interaction of Hac1p with a WD40 repeat-containing fragment of Cdc4p required the PEST sequence, and in particular Ser146, which is present within a sequence, TLS$_{146}$PKSMR, that fits the CPD motif (Nash *et al*., 2001). Ser146 is likely to be a site of phosphorylation by the kinase Srb10, whose activity was shown to be necessary for efficient degradation of Hac1p. Interaction of Hac1p with the Cdc4p fragment was partially inhibited by alanine substitution of S149, which lies just within the CPD. S149 is not present in the context of a CPD motif and is not a substrate for proline-directed kinases such as Srb10. Thus, the reduced interaction with Cdc4 observed with the S149A mutant is not due to the loss of an additional or alternative Cdc4 binding site. It is possible that phosphorylation of S149 by an as yet unknown kinase stimulates formation of the Cdc4 phospho degron by potentiating phosphorylation of S146 by Srb10 (or another kinase).

The regulation of turnover of Hac1p shares many features with that of the transcription factor Gcn4p, which in addition to its involvement as a master regulator of diverse responses to starvation and stress signals (Hinnebusch and Natarajan, 2002), has recently been shown to cooperate with Hac1p in activating UPRE-induced genes (Patil *et al*., 2004). Like Hac1p, Gcn4p is a bZIP protein whose cellular levels are regulated both by translational control of synthesis (Hinnebusch, 1984) and by degradation (Kornitzer *et al*., 1994). Like Hac1p, it is a highly unstable protein whose degradation by the proteasome requires Cdc34p and SCF^{Cdc4}, to which it is targeted by phosphorylation of one or more S/TP motifs by Srb10 or Pho85 kinases (Meimoun *et al*., 2000; Chi *et al*., 2001). Srb10 is implicated in the phosphorylation of Gcn4p during their encounter at promoter regions, whereas Pho85 is a regulator of Gcn4p in response to the availability of amino acids (for review, see Hinnebusch and Natarajan, 2002).

The presence in Hac1p of two potential phosphorylation sites in the CPD provides the opportunity for physiological regulation of its turnover. SCF complexes are known to couple protein kinase signaling pathways to the control of protein abundance. Petroski and Deshaies (2005) have reviewed three different modes of regulation for SCF-dependent proteolysis. The "AND" mode used for degradation of cyclin E by human SCF^{Cdc4} requires phosphorylation by both GSK3 and CDK kinases (Welcker *et al*., 2003). The "nanoswitch" or "threshold" mode of regulation is exemplified by SCF^{Cdc4}-mediated degradation of the yeast Sic1p CDK inhibitor, which requires phosphorylation by Cdc28p of any six of nine potential sites that are present within suboptimal CDP sequences (Nash *et al*., 2001; Orlicky *et al*., 2003). Finally, SCF^{Cdc4}-mediated degradation of Gcn4 illustrates the "OR" mode of control because it requires CPD phosphorylation by either Srb10 or Pho85 (Meimoun *et al*., 2000; Chi *et al*., 2001). Recently, another OR mode, involving a protein having two or more degrons that are regulated by different SCF complexes, has been described for Gal4p (Muratani *et al*., 2005). In this case, SCF^{Grr1} facilitates turnover of Gal4p at a relatively slow rate during yeast cell growth in the presence of a noninducing carbon source, whereas SCF$^{Dsg1/Mdm30}$ mediates rapid degradation of a differentially phosphorylated form of Gal4p during growth in the presence of galactose (Muratani *et al*., 2005). Regulation of Hac1p degradation does not involve the AND or nanoswitch modes, because it contains only a single CPD that does not require simultaneous activation by more than one kinase, although as discussed above, phosphorylation of a second nearby site may stimulate the interaction of the CPD with Cdc4p. We have not ruled out the possibility that the classical "OR" mode of regulation for SCF-dependent proteolysis may apply to Hac1p, with one or more additional kinases capable of targeting Ser146 to activate SCF^{Cdc4}-mediated degradation of Hac1p.

The PEST/CPD degron that we have identified in Hac1p does not fully account for the extremely rapid rate of degradation of Hac1p. Point mutation or deletion of this degron, defects in components of the SCF^{Cdc4} complex and deletion of the Srb10 kinase all cause significant stabilization of Hac1p. However, even after the increase in half-life from ~1 to 4–5 min, Hac1p is still a rapidly turned over protein. In a separate study, we have identified a second degron in Hac1p that is colocalized with the transactivation domain at the C terminus of the protein (Pal, Dyke, and Gething, unpublished data).

There are many examples of unstable transcription factors in which there is overlap between TAD and degron sequences (Salghetti *et al*., 2000; Muratani and Tansey, 2003). Such overlap is thought to contribute to the coupling of the activity of transcription factors with their proteasome-mediated destruction and various models have been proposed to

describe how activator degradation might limit or stimulate transcription (Salghetti *et al*., 2000; Lipford and Deshaies, 2003; Muratani and Tansey, 2003; Lipford *et al*., 2005). Thus, degradation might function to limit how long any single activator remains bound at a promoter, facilitating reprogramming of transcriptional patterns, or it might be required to remove spent activators that would otherwise block continued initiation of transcription. Alternatively, degradation may be linked to prior ubiquitin-mediated "licencing" of transcription factor activity. In Hac1p, not only is the C-terminal degron colocalized with the TAD, but the function of the PEST degron/CPD, which is distant from the TAD in the primary sequence of the protein, may also be dependent on transcription because it is inhibited in cell mutants lacking Srb10 kinase, a component of the SRB/mediator module of the RNA polymerase II holoenzyme.

Modulation of the rate of degradation of Hac1p would be of little consequence if it did not affect the pattern of transcription of UPRE-regulated genes. Transactivation of a reporter construct that is under the control of the UPRE derived from the *KAR2* (BiP) gene (Mori *et al*., 1993) was increased approximately twofold by blockage of SCF^{Cdc4}-mediated degradation of Hac1p. Transactivation of other UPR-controlled genes is likely to be up-regulated to much greater extents, because *KAR2* belongs to a subclass of UPRE-controlled genes whose transcription seems to be maximally activated at low concentrations of Hac1p (Leber *et al*., 2004). As noted by Leber *et al*. (2004), target genes of this class likely have promoters that are saturated by the lower amount of Hac1p and thus reach full activation more readily. For a variety of other UPR targets, induction continues to increase as Hac1p levels increase; lower concentrations of Hac1p are inadequate for full stimulation of these genes, which may have lower affinity for Hac1p. Consistent with this, cell viability under ER stress conditions was increased up to 50-fold by point mutation or deletion of the Hac1p phospho degron, or as the consequence of defects in components of the SCF^{Cdc4}-dependent proteasomal degradation pathway. Leber *et al*. (2004) also commented that because genes respond differentially to Hac1p levels, regulation of *HAC1* mRNA abundance can be used as a gene-specific gain control for target activation. We propose that regulation of Hac1p stability could serve a similar purpose, especially if other transcription factors, such as Gcn4p (Patil *et al*., 2004) or UMF (Leber *et al*., 2004), that collaborate with Hac1p to up-regulate the UPR are simultaneously stabilized. That the same SCF^{Cdc4} machinery is used for turnover of Hac1p and Gcn4p provides the opportunity for their coregulation via physiological inputs that target this machinery.

We recently identified a physiological condition that could regulate the UPR via stabilization of Hac1p by showing that overexpression of components of the cell integrity (CI) Pkc1p–MAP kinase signaling pathway suppress the transcriptional defect of a reporter gene controlled by a point mutated UPRE element (Helfenbaum, unpublished data). Activation of the CI pathway, which responds to weakening or stretching of the cell wall and functions to activate the synthesis and delivery of cell wall components (Gustin *et al*., 1998; Heinisch *et al*., 1999), increases both the rate of ER stress-mediated induction of *HAC1* mRNA splicing (Helfenbaum, unpublished data) and the stability of Hac1p, suggesting that the multicopy suppression occurred because the increased level of accumulation of Hac1p compensated for its reduced affinity for the point mutated UPRE element. Analysis of the roles played by the CI pathway kinases indicated that Pkc1p was necessary for acceleration of *HAC1* mRNA splicing under hypotonic shock conditions that activate CI signaling (Helfenbaum, unpublished data), whereas both Pkc1p and Mpk1p were required for stabilization of Hac1p even under normal (isotonic) conditions. Although it is intriguing that the sequence surrounding Ser146 in Hac1p provides phosphorylation motifs for both Pkc1p and Mpk1p, our data are not compatible with one or both of these kinases playing a direct role in regulating degradation of Hac1p by targeting this residue. Phosphorylation of Ser146 should activate SCF-mediated degradation of Hac1p; however, CI pathway activation of Pkc1p and Mpk1p stabilizes Hac1p and accumulation of Hac1p is significantly decreased in Δ*pkc1* or Δ*mpk1* cells. Furthermore, the overall level of phosphorylation of Hac1p is increased, rather than decreased, in Δ*pkc1* and Δ*mpk1* cells, suggesting that a complex interplay between kinases and phosphatases is likely to be involved in the regulation of phosphorylation of Hac1p. In the specific context of regulation of SCF^{Cdc4}-dependent degradation of Hac1p, it is possible that the CI kinases act indirectly by inhibiting other kinase(s), such as Srb10, that phosphorylate Ser146 to target Hac1p for destruction, or by activating a phosphatase that reverses phosphorylation at Ser146.

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