D box and KEN box motifs in budding yeast Hsl1p are required for APC-mediated degradation and direct binding to Cdc20p and Cdh1p

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The precise order of molecular events during cell cycle progression depends upon ubiquitin-mediated proteolysis of cell cycle regulators. We demonstrated previously that Hsl1p, a protein kinase that inhibits the Swe1p protein kinase in a bud morphogenesis checkpoint, is targeted for ubiquitin-mediated turnover by the anaphase-promoting complex (APC). Here, we investigate regions of Hsl1p that are critical both for binding to the APC machinery and for APC-mediated degradation. We demonstrate that Hsl1p contains both a destruction box (D box) and a KEN box motif that are necessary for Hsl1p turnover with either APC\textsuperscript{Cdc20} or APC\textsuperscript{Cdh1}. In coimmunoprecipitation studies, the D box of full-length Hsl1p was critical for association with Cdc20p, whereas the KEN box was important for association with Cdh1p. Fusion of a 206-amino-acid fragment of Hsl1p containing these motifs to a heterologous protein resulted in APC-dependent degradation of the fusion protein that required intact D box and KEN box motifs. Finally, this bacterially expressed Hsl1p fusion protein interacted with Cdc20p and Cdh1p either translated in vitro or expressed in and purified from insect cells. Binding to Cdc20p and Cdh1p was disrupted completely by a D box/KEN box double mutant. These results indicate that D box and KEN box motifs are important for direct binding to the APC machinery, leading to ubiquitination and subsequent protein degradation.

Key Words: Cdc20p; Cdh1p; KEN box; destruction box; ubiquitination; anaphase-promoting complex

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Ubiquitin-mediated protein degradation is necessary for the linear progression of molecular events during the cell cycle. Protein ubiquitination and subsequent degradation by the 26S proteasome is required for the initiation of DNA replication, the onset of anaphase, and for mitotic exit (for reviews, see King et al. 1996; Peters 1999; Zachariae and Nasmyth 1999). The ubiquitin pathway is a multi-step process in which the 76-amino-acid ubiquitin molecule is activated by an E1 enzyme, transferred to an E2 enzyme, and then attached covalently to the protein substrate either directly or in conjunction with an E3 enzyme [ubiquitin ligase] (for reviews, see Ciechanover 1994; Hochstrasser 1996). Proteins destined to be degraded are subject to multiple rounds of ubiquitin attachment [polyubiquitination] and are then proteolyzed by the 26S proteasome. The E3 or ubiquitin ligase is involved in protein substrate recognition and thereby confers specificity to the ubiquitination reaction (for reviews, see Ciechanover 1994; Hochstrasser 1996).

The SCF \{Skp1p–cullin–F-box\} ubiquitin ligases are a family of multi-subunit E3s that are responsible for ubiquitination of cell cycle regulators at the G\textsubscript{1}–S phase transition (for reviews, see King et al. 1996; Krek 1998; Peters 1998). SCF complexes have common Skp1 and cullin subunits, but distinct F-box protein subunits that contain either a WD40 domain or a leucine-rich repeat domain that binds directly to phosphorylated SCF substrates (for reviews, see Krek 1998; Patton et al. 1998; Peters 1998). In \textit{Saccharomyces cerevisiae}, the G\textsubscript{1}-cyclins, Cln1p and Cln2p, bind SCF\textsuperscript{Grr1} \{Deshaies et al. 1995; Skowyra et al. 1999\}, whereas the Cdc28p kinase inhibitor, Sic1p, has been shown to bind SCF\textsuperscript{Cdc4} \{Feldman et al. 1997; Skowyra et al. 1997; Verma et al. 1997\}, where Grr1p and Cdc4p are the F-box components of these SCFs.

The anaphase-promoting complex \{APC\}, or cyclosome, another multi-subunit E3, is responsible for the ubiquitination of cell regulators at the metaphase–anaphase–G\textsubscript{2} transitions (for review, see Zachariae and Nasmyth 1999). The APC is comprised of a core complex of proteins and two WD40-containing proteins, Cdh1 [called Cdh1p or Hct1p in \textit{S. cerevisiae}] and Cdc20, that bind to, and have been proposed to act-
tivate, the APC [Schwab et al. 1997; Visintin et al. 1997; Fang et al. 1998b; Kramer et al. 1998; Lim et al. 1998; Zachariae et al. 1998; Kotani et al. 1999]. Cdc20 binds the APC (APC\textsuperscript{Cdc20}) during mitosis. APC\textsuperscript{Cdc20} is required for the degradation of the anaphase inhibitors known as the securins [Pds1p in \textit{S. cerevisiae} and Cut2p in \textit{Schizosaccharomyces pombe}], thereby triggering sister chromatid separation [Cohen-Fix et al. 1996; Funabiki et al. 1996; Stratmann and Lehrer 1996; Yamamoto et al. 1996; Ciosk et al. 1998; Zou et al. 1999; Zur and Brandeis 2001]. Cdh1 binds to the APC (APC\textsuperscript{Cdh1}) in late mitosis and G1, and is responsible for ubiquitination of the mitotic cyclins [Clb1–4p in \textit{S. cerevisiae}] during these cell cycle phases [Schwab et al. 1997; Visintin et al. 1997; Kramer et al. 1998; Zachariae et al. 1998; Jaspersen et al. 1999]. APC\textsuperscript{Cdh1} is also responsible for the degradation of Cdc20, thereby restricting APC\textsuperscript{Cdc20} activity to a narrow window from G2 when Cdc20 is synthesized to late mitosis when Cdc20 is degraded by APC\textsuperscript{Cdh1} [Fang et al. 1998b; Prinz et al. 1998; Shirayama et al. 1998]. In contrast, Cdh1 levels remain relatively constant during the cell cycle, but phosphorylation of Cdh1 by the mitotic kinase Cdc2 [Cdc28p in \textit{S. cerevisiae}] blocks Cdh1 binding to the APC, thereby preventing APC\textsuperscript{Cdh1} activity during S, G2, and M phases when Cdc2/Cdc28p activity is high [Zachariae et al. 1998; Jaspersen et al. 1999; Shirayama et al. 1999; Blanco et al. 2000; Listovsky et al. 2000].

Two degradation motifs have been identified in APC substrates. The destruction box (D box), with the consensus sequence R-x-x-L-x-x-x-N/D/E, is important for the degradation of most APC substrates [Glotzer et al. 1991; for review, see Zachariae and Nasmyth 1999]. Recently, human Cdc20 (hCdc20), which lacks a D box, was found to contain a new degradation signal called a ‘KEN box’ with the consensus sequence K-E-N-x-x-x-(Pfleger and Kirschner 2000). Subsequently, a few other APC substrates containing both KEN box and D box motifs have been identified. These APC substrates are human CDC6, a protein required for DNA replication, human securin, and the mitotic cyclin A in \textit{Drosophila melanogaster} [Peterson et al. 2000; Jacobs et al. 2001; Zur and Brandeis 2001]. Functional KEN boxes in yeast proteins have not yet been reported.

Because direct interactions between Cdc20 or Cdh1 with APC substrates have not been demonstrated, generally these proteins are believed to activate the APC, possibly by inducing allosteric changes in core APC subunits leading to specific substrate binding and ubiquitination. However, indirect evidence for substrate binding has led others to suggest, by analogy to the F-box proteins of the SCFs, that Cdc20 and Cdh1 may bind and target protein substrates to the APC [Schwab et al. 1997; Visintin et al. 1997; Shirayama et al. 1998; Burton and Solomon 2000; Ohtoshi et al. 2000; Sorensen et al. 2001].

We found previously that Hsl1p, a 170-kD budding yeast protein kinase that negatively regulates the Swe1p protein kinase in a bud morphogenesis checkpoint pathway [Lew and Reed 1995; Barral et al. 1999; McMillan et al. 1999, Shulewitz et al. 1999], is degraded via the APC [Burton and Solomon 2000]. Like most APC-substrates, Hsl1p contains a D box that is important for its degradation. Hsl1p associates with both Cdc20p and Cdh1p based on both two-hybrid assays and coimmunoprecipitation from yeast extracts, indicating that it might interact directly with these proteins [Burton and Solomon 2000]. Given these findings, we believed that Hsl1p might serve as a useful model substrate for understanding how proteins destined for degradation are recognized by the APC.

In this study we set out to understand which domains of Hsl1p are important for both recognition and turnover by the APC machinery. We noticed that Hsl1p contains a potential KEN box and were interested in addressing the following questions using Hsl1p as a model APC substrate: 1) Do KEN boxes serve as degradation signals in yeast? 2) Can Hsl1p bind directly to Cdc20p and/or Cdh1p? 3) What is the role of the D box and/or KEN box in APC recognition? We discuss a model for substrate recognition by the APC machinery.

## Results

**Hsl1p degradation requires intact D box and KEN box motifs**

Previously, we demonstrated that the APC-dependent degradation of Hsl1p requires an intact destruction box (D box) motif [Burton and Solomon 2000]. Following the recent identification of the KEN box (KEN\textsubscript{XXX}N/D; bold indicates conserved and mutated residues) as a second APC-degradation signal important for APC\textsuperscript{Cdh1}-mediated degradation [Pfleger and Kirschner 2000], we scanned the Hsl1p sequence and found a potential KEN box motif that closely matched that of hCDC20 [Fig. 1A]. To determine if this sequence influences APC-mediated degradation of Hsl1p, the conserved amino acid residues (underlined in Fig. 1A) were mutated to alanines. The stabilities of full length Hsl1p–HA or of Hsl1p–HA containing a mutated D box [RAALSDITN to AAAASDTA] (Hsl1p\textsuperscript{mdb–HA}), a mutated KEN box (Hsl1p\textsuperscript{mkb–HA}), or both mutations (Hsl1p\textsuperscript{mdb/mkb–HA}) were investigated in G1, a stage of the cell cycle when APC\textsuperscript{Cdh1} is active. Cells were arrested in G1 with α-factor and induced to express the different isoforms of Hsl1p–HA by galactose induction. Then, expression was terminated by the addition of glucose and cycloheximide to the medium. Levels of the different forms of Hsl1p–HA were monitored by immunoblot analysis with anti-HA antibodies [Fig. 1B]. As shown previously [Burton and Solomon 2000], wild-type Hsl1p–HA was unstable in the presence of APC\textsuperscript{Cdh1}, but stable in a strain \textit{cdc23-1} mutated for a core APC subunit [Fig. 1B, top row]. In contrast, Hsl1p\textsuperscript{mdb–HA} containing the mutated D box was relatively stable even in the presence of an active APC [Fig. 1B, second row, left panel]. Similarly, mutation of the putative KEN box (Hsl1p\textsuperscript{mkb–HA}) or of both motifs (Hsl1p\textsuperscript{mdb/mkb–HA}) also resulted in Hsl1p stabilization [Fig. 1B, third and
fourth rows, left panels). These results indicate that KEN boxes are recognized by the APC machinery in yeast and that both a D box and a KEN box are necessary for maximal rates of Hsl1p degradation by APC\textsuperscript{Cdh1}.

Given the similarities between Cdh1p and Cdc20p, we were interested in whether APC\textsuperscript{Cdc20} could also mediate Hsl1p degradation. Although normally Hsl1p is degraded late in mitosis (Burton and Solomon 2000), we found that Hsl1p–HA was stable in anaphase-arrested cells expressing a nondegradable form of Clb2p (data not shown). Therefore, we assessed Hsl1p stability in G\textsubscript{1}, a point in the cell cycle at which Hsl1p is known to be unstable (Fig. 1B; Burton and Solomon 2000). In order to eliminate APC\textsuperscript{Cdh1} activity and look only at APC\textsuperscript{Cdc20} activity in G\textsubscript{1}, we used cdh1\textsuperscript{-}/H9004 cdc28-13 cells with or without an integrated copy of GAL–CDC20–myc. cdh1\textsuperscript{-}/H9004 cells are viable, but have elevated levels of Clb2p, resulting in an inefficient G\textsubscript{1} arrest with a-factor (Schwabet al. 1997; Visintin et al. 1997). The conditional cdc28-13 allele was used to arrest cells in G\textsubscript{1} by shifting the cells to 37°C to inactivate the APC in cdc23-1 strains. Proteins levels were monitored by immunoblotting with anti-HA antibodies at the indicated times following termination of Hsl1p expression.

Hsl1p degradation motifs influence association with the APC machinery

Next, we examined the importance of the D box and the KEN box for the association of Hsl1p with Cdc20p and Cdh1p in a coimmunoprecipitation assay. We showed previously that the D box in Hsl1p was important for association with Cdc20p, but not with Cdh1p (Burton and Solomon 2000). Full-length Hsl1p–HA, Hsl1p\textsuperscript{mdb–} and Hsl1p\textsuperscript{mkb–}HA were coimmunoprecipitated from G\textsubscript{1}-arrested cell lysates with Cdc20–myc (Fig. 2B), but not with Cdh1p (data not shown). Further, Hsl1p\textsuperscript{mdb–} and Hsl1p\textsuperscript{mkb–}HA were coimmunoprecipitated from G\textsubscript{1}-arrested cell lysates with both Cdc20–myc and Cdh1p (Fig. 2B). These results indicate that Hsl1p degradation can be mediated both by APC\textsuperscript{Cdc20} and by APC\textsuperscript{Cdh1} and that both D box and KEN box motifs are required in both cases.

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Figure 1. Hsl1p has both a D box and a KEN box and both are necessary for APC-mediated degradation in G\textsubscript{1}. (A) Hsl1p has a putative KEN box located at amino acids 775–781. The human CDC20 (hCDC20) KEN box and consensus sequence (Pfleger and Kirschner 2000) are shown for comparison. (B) APC\textsuperscript{Cdh1}-mediated degradation of Hsl1p–HA in G\textsubscript{1}-arrested cells requires both a D box and a KEN box. Cells were arrested in G\textsubscript{1} with a-factor [100 ng/mL] and induced to express GAL–HSL1–HA [YJB123 and YJB125], GAL–HSL1\textsuperscript{mdb–}HA [YJB229 and YJB270], GAL–HSL1\textsuperscript{mkb–}HA [YJB257 and YJB271] and GAL–HSL1\textsuperscript{mdb/mkb–}HA [YJB258 and YJB272] by the addition of galactose. Cells were shifted to 37°C to inactivate the APC in cdc23-1 strains. Proteins levels were monitored by immunoblotting with anti-HA antibodies at the indicated times following termination of Hsl1p expression.
HA, Hsl1p\(^{m\text{db}}\)-HA, or Hsl1p\(^{m\text{kb}}\)-HA were co-overexpressed in yeast with either GST–Cdc20p or GST–Cdh1p. The different Hsl1p–HA proteins were then immunoprecipitated (IP) from cell extracts using anti-HA antibodies. Coimmunoprecipitation of GST–Cdc20p or GST–Cdh1p was examined by immunoblot analysis with anti-GST antibodies (Fig. 3A). As found previously, both GST–Cdc20p and GST–Cdh1p coimmunoprecipitated with wild-type Hsl1p–HA (Fig. 3A, lower panels, lanes 2 and 7), whereas little or no GST–Cdc20p or GST–Cdh1p was detected in control immunoprecipitations from strains lacking an HA-tag (Fig. 3A, lower panels, lanes 1 and 6). Mutation of the D box in Hsl1p compromised association with Cdc20p, but had little effect on Cdh1p association (Fig. 3A, lower panels, lanes 3 and 8; see also Figs. 5B, 6, and 8C, below). In contrast, mutation of the KEN box had little effect on Cdc20p association, but eliminated Cdh1p association (Fig. 3A, lower panels, lanes 4 and 9). Neither GST–Cdc20p nor GST–Cdh1p associated with Hsl1p\(^{m\text{db/mkb}}\) (Fig. 3A, lower panels, lanes 5 and 10). These results indicate that in the context of full-length Hsl1p–HA, the D box is important for association with Cdc20p, whereas the KEN box is important for association with Cdh1p. Since both motifs must be present for efficient degradation (Figs. 1B and 2A), these results also indicate that association, though necessary, is not sufficient to promote degradation. Though suggestive, it is important to note that these data do not show direct binding of Hsl1p to Cdc20p and Cdh1p. It is possible that another yeast protein, such as a core APC subunit, serves as a bridge between Hsl1p and Cdc20p or Cdh1p in the immunoprecipitates.

To test the generality of these findings, we tested whether Clb2p, a mitotic cyclin that is a well-documented APC substrate (Irniger et al. 1995; Schwab et al. 1997; Jaspersen et al. 1998; Zachariae et al. 1998) could associate also with Cdc20p and Cdh1p by coimmunoprecipitation studies. Clb2p–HA or Clb2p\(^{m\text{db}}\)-HA were co-overexpressed with GST–Cdc20p or GST–Cdh1p and analyzed as for Hsl1p–HA (Fig. 3B). Mutation of this D box stabilizes Clb2p (Irniger et al. 1995; Schwab et al. 1997; Jaspersen et al. 1998; Zachariae et al. 1998). Clb2p–HA was able to coimmunoprecipitate both GST–Cdc20p and GST–Cdh1p (Fig. 3B, lower panels, lanes 2 and 5), but these proteins were not present in immunoprecipitations from strains lacking an HA-tag (Fig. 3B, lower panels, lanes 1 and 4). However, coimmunoprecipitation of GST–Cdc20p and GST–Cdh1p was not affected by mutation of the Clb2p D box (Fig. 3B, lower panels, lanes 3 and 6). It is possible that a recently identified KEN box in Clb2p (S. Holloway, pers. comm.) may mediate association of Clb2p\(^{m\text{db}}\) with Cdc20p and Cdh1p. It is also possible that the interaction we observed between Cdc20p and Clb2p may reflect phosphorylation of Cdc20p by Cdk2, as occurs with CDK2 and human CDC20 (Ohtoshi et al. 2000). These results indicate that the association of APC substrates with Cdc20p and Cdh1p is not unique to Hsl1p and that other APC substrates might associate with these proteins, although the requirements for the interactions may differ.
Hsl1p667–872 can act as a transposable degradation signal

We investigated whether fragments of Hsl1p containing both the KEN box and the D box, located at amino acids 775–781 and 828–836, respectively, could serve as portable degradation signals. We started with a 206-amino-acid fragment containing both motifs plus 108 amino acids N-terminal to the KEN box (Hsl1p667–872; Fig. 4, top row, left panel). The upstream residues were included because of the observation that APC-mediated degradation of hCDC20 requires amino acid residues upstream of the KEN box (Pfleger and Kirschner 2000). Hsl1p667–872 was fused to the C terminus of the E. coli maltose binding protein (MBP) and expressed in yeast. The stabilities of this fusion protein and of progressively smaller fusion proteins comprising the KEN box and D box were tested in G1-arrested cells to examine APCCdh1p-mediated degradation (Fig. 4). MBP alone was found to be stable when expressed in yeast cells (data not shown). MBP–Hsl1p667–872 was very unstable and was undetectable 15 min after terminating its synthesis (Fig. 4, top row, middle panel). The upstream residues were included because of the observation that APC-mediated degradation of hCDC20 requires amino acid residues upstream of the KEN box (Pfleger and Kirschner 2000). Hsl1p667–872 was fused to the C terminus of the E. coli maltose binding protein (MBP) and expressed in yeast. The stabilities of this fusion protein and of progressively smaller fusion proteins comprising the KEN box and D box were tested in G1-arrested cells to examine APCCdh1p-mediated degradation (Fig. 4). MBP alone was found to be stable when expressed in yeast cells (data not shown). MBP–Hsl1p667–872 was very unstable and was undetectable 15 min after terminating its synthesis (Fig. 4, top row, middle panel). The instability of MBP–Hsl1p667–872 was due to APC-mediated degradation as the protein was stable in cdc23-1 cells (Fig. 4, top row). MBP–Hsl1p667–872, which removes 35 amino acid residues downstream of the D box, was also quite unstable; this instability was APC-dependent (Fig. 4, second row). However, truncation of the N-terminal domain upstream of the KEN box greatly stabilized the MBP–Hsl1p fusion proteins [MBP–Hsl1p701–872, MBP–Hsl1p740–872, and MBP–Hsl1p764–872; Fig. 4, rows 3–7]. These results suggest that sequences upstream of the KEN box motif are important for degradation of the fusion protein, although it is not yet clear if this region contains an additional degradation motif or if it provides a structural requirement for KEN box recognition. These results show that fragments of Hsl1p can act as transposable degradation signals when fused to MBP. Based on its rapid degradation, we chose MBP–Hsl1p667–872 for further analysis.

First, we wanted to confirm that the APC-mediated degradation of MBP–Hsl1p667–872 was D box- and KEN box-dependent. The stabilities of MBP–Hsl1p667–872, MBP–Hsl1p667–872mdb, MBP–Hsl1p667–872mkb, and MBP–Hsl1p667–872mdb/mkb were monitored in G1-arrested cells as described for full-length Hsl1p. Mutation of either the D box or KEN box resulted in stabilization of MBP–Hsl1p667–872 (Fig. 5A, mdb and mkb) and mutation of both motifs resulted in complete stabilization of the fusion protein (Fig. 5A, mdb/mkb). These findings indicate that APCCdh1p-dependent degradation of MBP–Hsl1p667–872 and Hsl1p–HA both require a D box and a KEN box.

Next, we tested whether MBP–Hsl1p667–872, like Hsl1p, could associate with Cdc20p and Cdh1p by coimmunoprecipitation. The different forms of MBP–Hsl1p667–872 were co-overexpressed with either GST–Cdc20p or GST–Cdh1p. The MBP–Hsl1p667–872 fusions were immunoprecipitated and the levels of coimmunoprecipitating GST–Cdc20p or GST–Cdh1p were analyzed by immunoblot analysis [Fig. 5B, lower panels]. We
found that GST–Cdc20p had the same specificity of interaction with MBP–Hsl1p667–872 as was observed for full-length Hsl1p–HA [cf. Fig. 5B lower panel, lanes 1–5 with Fig. 3A]. Mutation of the D box or of both the D box and the KEN box prevented the communoprecipitation of GST–Cdc20p (Fig. 5B, lower panel, lanes 3 and 5), whereas GST–Cdc20p associated equally well with the wild-type and the KEN box mutant forms of MBP–Hsl1p667–872 (Fig. 5B, lower panel, lanes 2 and 4). In contrast, GST–Cdh1p did not associate with MBP–Hsl1p667–872 (Fig. 5B, lower panel, lanes 7–10), indicating either that Cdh1p cannot associate well with this domain of Hsl1p, or that the combination of the MBP and GST tags and anti-MBP–antibodies interferes with the association. We favor the latter possibility because MBP–Hsl1p667–872 is degraded in G1-arrested cells in which

**Figure 4.** Analysis of Hsl1p sequences that can act as transposable APC-dependent degradation signals when fused to the carboxy terminus of the maltose binding protein (MBP). Left panels, schematic representations of amino acids of Hsl1p containing the KEN box and D box motifs (shaded boxes) that were fused to MBP (data not shown). Middle and right panels, stability of MBP–Hsl1p proteins in G1-arrested cells. Wild-type and cdc23–1 cells were arrested in G1 with α-factor [100 ng/mL] and then induced to express GAL–MBP–HSL1667–872 (YJB306 and YJB311), GAL–MBP–HSL167–837 (YJB320 and YJB331), GAL–MBP–HSL1701–872 (YJB321 and YJB335), GAL–MBP–HSL1701–837 (YJB329 and YJB336), GAL–MBP–HSL1740–872 (YJB322 and YJB332), GAL–MBP–HSL1740–837 (YJB330 and YJB337) and GAL–MBP–HSL1764–872 (YJB307 and YJB311) fusions by the addition of galactose. Strains were subsequently shifted to 37°C to inactivate the APCCdh1 activity predominates [Figs. 4A, 5A] and because of direct binding data between MBP–Hsl1p667–872 and Cdh1p presented below.

**Cdc20p and Cdh1p bind directly to Hsl1p667–872 in a D box– and KEN box-dependent manner**

To test whether Hsl1p interacts directly with Cdc20p and Cdh1p, we used recombinant MBP–Hsl1p667–872 produced in *E. coli* and purified on amylose resin and [35S]methionine-labeled Cdc20p and Cdh1p translated in vitro in reticulocyte lysates (Fig. 6). MBP and the various MBP–Hsl1p proteins on amylose resin were incubated with [35S]-labeled Cdc20p and Cdh1p and washed. The bound proteins were run on SDS-polyacrylamide gels and analyzed for Cdc20p or Cdh1p by fluorography (Fig. 6, top panels). [35S]-labeled Cdc20p bound to MBP–Hsl1p667–872 and MBP–Hsl1p667–837 but not to MBP alone or to MBP–Hsl1p667–872mkb (Fig. 6, top, left panel, lanes 1–4). [35S]-labeled Cdh1p exhibited a similar binding profile for the different forms of MBP–Hsl1p667–872 (Fig. 6, top panels, cf. lanes 6–8 with lanes 2–4). Similar amounts of the recombinant MBP–Hsl1p667–872 proteins were present on the amylose beads, as visualized by Coomassie staining [Fig. 6, lower panels]. Currently, we do not know why mutation of the KEN box has less of an effect on the association of MBP–Hsl1p667–872 with Cdh1p (see also below), than on that of full-length Hsl1p with Cdh1p. We used purified proteins to rule out the possibility that the interaction of Hsl1p with these proteins was mediated by a core APC subunit or other protein from the reticulocyte lysate. We used purified Cdh1p–6xHis recombinant protein from insect cells (Jaspersen et al. 1999) in binding assays with MBP–Hsl1p667–872. Extracts from uninfected control cells or from cells infected with a baculovirus encoding Cdh1p–6xHis were incubated with Talon metal affinity resin (Clonetech) to purify Cdh1p–6xHis. A band corresponding to Cdh1p–6xHis was observed (Fig. 7A, lane 7 arrow) in the sample derived from baculovirus-infected cells that migrated at the appropriate molecular weight and was absent in the uninfected cell control (Fig. 7A, cf. lanes 6 and 7). Extracts from *E. coli* cells expressing MBP, MBP–Hsl1p667–872, MBP–Hsl1p667–872mkb, MBP–Hsl1p667–872mbk, or MBP–Hsl1p667–872mkb were prepared (Fig. 7A, lanes 1–5). The different *E. coli* extracts were incubated either with resin bound to Cdh1p–6xHis or with beads incubated with uninfected cell extracts. Beads were washed and bound proteins were examined by Coomassie staining of SDS-polyacrylamide gels (Fig. 7B). Protein bands corresponding to the MBP–Hsl1p667–872 fusion proteins were observed clearly in eluates from the Cdh1p–6xHis beads, but not with the uninfected cell bead eluates (Fig. 7B, cf. lanes 2–4 with lanes 6–8). MBP–Hsl1p667–872mkb appeared to bind Cdh1p somewhat less well than MBP–Hsl1p667–872 (Fig. 7B, cf. lane 3 with lane 2). The nonspecific binding of MBP to both of the resins (Fig. 7B, lanes 1 and 5) is likely due to the massive
amounts of MBP in the E. coli lysates (Fig. 7A, cf. lane 1 with lanes 2–5). Given the high stoichiometry of MBP–Hsl1p–Cdh1p binding, it is unlikely that their association is mediated by a contaminant in the Cdh1p–6xHis preparation because these are all present at very low stoichiometries relative to Cdh1p–6xHis and are also present in the preparation from uninfected cells (Fig. 7A lanes 6 and 7).

We improved the sensitivity of this assay to D box and KEN box mutations by diluting the E. coli lysates sufficiently (20-fold) that binding of MBP–Hsl1p667–872 proteins began to decline. We also reduced the amount of MBP alone (by 320-fold) so that it was similar to the amount of the MBP–Hsl1p667–872 fusion proteins used. By Coomassie staining we observed binding of MBP–Hsl1p667–872 and MBP–Hsl1p667–872mkb to Cdh1p–6xHis (Fig. 8A, lanes 2 and 4). A faint band corresponding to MBP–Hsl1p667–872mdb was detected by Coomassie staining and confirmed by immunoblot analysis (Fig. 8A, lane 3). Mutation of both the D box and the KEN box abolished detectable binding to Cdh1p–6xHis completely (Fig. 8A, lane 5). MBP and the MBP–Hsl1p667–872 proteins began to decline. We also reduced the amount of MBP alone (by 320-fold) so that it was similar to the amount of the MBP–Hsl1p667–872 fusion proteins used. By Coomassie staining we observed binding of MBP–Hsl1p667–872 and MBP–Hsl1p667–872mkb to Cdh1p–6xHis (Fig. 8A, lanes 2 and 4). A faint band corresponding to MBP–Hsl1p667–872mdb was detected by Coomassie staining and confirmed by immunoblot analysis (Fig. 8A, lane 3). Mutation of both the D box and the KEN box abolished detectable binding to Cdh1p–6xHis completely (Fig. 8A, lane 5). MBP and the MBP–Hsl1p667–872 proteins began to decline. We also reduced the amount of MBP alone (by 320-fold) so that it was similar to the amount of the MBP–Hsl1p667–872 fusion proteins used. By Coomassie staining we observed binding of MBP–Hsl1p667–872 and MBP–Hsl1p667–872mkb to Cdh1p–6xHis (Fig. 8A, lanes 2 and 4). A faint band corresponding to MBP–Hsl1p667–872mdb was detected by Coomassie staining and confirmed by immunoblot analysis (Fig. 8A, lane 3). Mutation of both the D box and the KEN box abolished detectable binding to Cdh1p–6xHis completely (Fig. 8A, lane 5). MBP and the MBP–Hsl1p667–872 proteins began to decline. We also reduced the amount of MBP alone (by 320-fold) so that it was similar to the amount of the MBP–Hsl1p667–872 fusion proteins used. By Coomassie staining we observed binding of MBP–Hsl1p667–872 and MBP–Hsl1p667–872mkb to Cdh1p–6xHis (Fig. 8A, lanes 2 and 4). A faint band corresponding to MBP–Hsl1p667–872mdb was detected by Coomassie staining and confirmed by immunoblot analysis (Fig. 8A, lane 3). Mutation of both the D box and the KEN box abolished detectable binding to Cdh1p–6xHis completely (Fig. 8A, lane 5). MBP and the MBP–Hsl1p667–872 proteins began to decline. We also reduced the amount of MBP alone (by 320-fold) so that it was similar to the amount of the MBP–Hsl1p667–872 fusion proteins used. By Coomassie staining we observed binding of MBP–Hsl1p667–872 and MBP–Hsl1p667–872mkb to Cdh1p–6xHis (Fig. 8A, lanes 2 and 4). A faint band corresponding to MBP–Hsl1p667–872mdb was detected by Coomassie staining and confirmed by immunoblot analysis (Fig. 8A, lane 3). Mutation of both the D box and the KEN box abolished detectable binding to Cdh1p–6xHis completely (Fig. 8A, lane 5). MBP and the MBP–Hsl1p667–872 proteins began to decline. We also reduced the amount of MBP alone (by 320-fold) so that it was similar to the amount of the MBP–Hsl1p667–872 fusion proteins used. By Coomassie staining we observed binding of MBP–Hsl1p667–872 and MBP–Hsl1p667–872mkb to Cdh1p–6xHis (Fig. 8A, lanes 2 and 4). A faint band corresponding to MBP–Hsl1p667–872mdb was detected by Coomassie staining and confirmed by immunoblot analysis (Fig. 8A, lane 3). Mutation of both the D box and the KEN box abolished detectable binding to Cdh1p–6xHis completely (Fig. 8A, lane 5). MBP and the MBP–Hsl1p667–872 proteins began to decline. We also reduced the amount of MBP alone (by 320-fold) so that it was similar to the amount of the MBP–Hsl1p667–872 fusion proteins used. By Coomassie staining we observed binding of MBP–Hsl1p667–872 and MBP–Hsl1p667–872mkb to Cdh1p–6xHis (Fig. 8A, lanes 2 and 4). A faint band corresponding to MBP–Hsl1p667–872mdb was detected by Coomassie staining and confirmed by immunoblot analysis (Fig. 8A, lane 3). Mutation of both the D box and the KEN box abolished detectable binding to Cdh1p–6xHis completely (Fig. 8A, lane 5). MBP and the MBP–Hsl1p667–872 proteins began to decline.
Hsl1p667–872 proteins did not bind to the control uninfected cell resin (Fig. 8A,B, lanes 6–10).

Direct binding of Hsl1p667–872 to Cdc20p was tested using the diluted bacterial extracts expressing MBP or the different forms of MBP–Hsl1p667–872. (Right panel) 25 µL of cobalt resin from uninfected cell extracts and Cdh1p–6xHis baculovirus-infected Sf9 cell extracts. (B) Cobalt resin was incubated with extracts from insect cells infected with Cdh1p–6xHis baculovirus [lanes 1–4] or from uninfected cells [lanes 5–8]. Resins were washed and then incubated with 1 mL of the indicated E. coli extracts containing MBP or one of the MBP–Hsl1p667–872 fusion proteins and washed. Bound proteins were eluted with 150 mM imidazol and visualized by Coomassie staining following SDS-PAGE.

The D box and the KEN box form a bipartite degradation signal in Hsl1p

Hsl1p is a member of a growing subfamily of APC substrates that contain KEN boxes. The KEN box was first identified in human CDC20 and Nek2 and in mouse B99 as a motif essential for the Cdh1-dependent degradation of these proteins (Pfleger and Kirschner 2000). Subsequently, essential KEN boxes have been found in human CDC6 (Peterson et al. 2000), human securin (Zur and Brandeis 2001), Drosophila cyclin A (Jacobs et al. 2001), and now budding yeast Hsl1p. In these four cases, a functional D box is located within 53 amino acids of the KEN box (counting from the lysine of the KEN box to the arginine of the D box). A simple database search of budding yeast sequences reveals 92 proteins containing matches to both a basic D box [(RxxLxxxx[NDEQ]) and a basic KEN box (KENxxxx[NDEQ]), in 32 of which the two motifs are separated by fewer than 100 amino acids. Although only some of these proteins will be authentic APC substrates, the exercise indicates that dual recognition may be a common theme [discussed in more detail below]. It is interesting to note that Cut2p and Pds1p, the securin proteins in fission and budding yeast, respectively, each contain a potential KEN box within the first 15 amino acids of the protein, situated upstream of their characterized D boxes. This positioning is virtually identical to that of the characterized KEN box in human securin (Zur and Brandeis 2001), indicating that the positioning of these motifs is criti-
cal, despite the low overall similarity between these proteins.

Both the D box and the KEN box are essential for efficient Hsl1p degradation by both APC\textsuperscript{Cdc20} and APC\textsuperscript{Cdh1}. The efficient degradation of Hsl1p in G\textsubscript{1} cells containing only one form of the APC [either APC\textsuperscript{Cdh1}, Figs. 1 and 5, or APC\textsuperscript{Cdc20}, Fig. 2] indicates that these signals are recognized in concert, not by parallel pathways. The ability of both APC\textsuperscript{Cdc20} and APC\textsuperscript{Cdh1} to recognize both motifs suggests that many proteins will be substrates for both forms of the APC, although at different times. For Hsl1p it is likely that APC\textsuperscript{Cdc20} initiates Hsl1p degradation during mitosis, whereas any remaining Hsl1p may be degraded by APC\textsuperscript{Cdh1} during mitotic exit and entry into G\textsubscript{1}. In addition to temporal control, it is possible that differences in substrate specificity between these APC forms could arise from differences in affinity for particular degradation motifs within a substrate, or from weak interactions with other regions of the substrate.

The interplay between the D box and the KEN box is not as clear for the other characterized KEN box-containing proteins. Human CDC20 does not contain an obvious D box and is degraded solely by APC\textsuperscript{Cdh1} (Pfleger and Kirschner 2000). No D box was reported for either Nek2 or B99, both of which can be degraded by APC\textsuperscript{Cdh1}, although it was not reported whether they can also be degraded by APC\textsuperscript{Cdc20} (Pfleger and Kirschner 2000). These studies led to the suggestion that the KEN box was required for recognition by APC\textsuperscript{Cdh1} but not APC\textsuperscript{Cdc20}. Intriguingly, potential D boxes have been identified in both Nek2 and B99 (C. Pfleger and M. Kirschner, pers. comm.). In human CDC6, mutation of either the D box or the KEN box could stabilize CDC6 partially, whereas the double mutant was stabilized fully (Peterson et al. 2000). For Drosophila cyclin A, both motifs must be intact for full instability (Jacobs et al. 2001). In contrast, either motif was capable of destabilizing human securin (Zur and Brandeis 2001). Human CDC6, Drosophila cyclin A, and human securin were reported to be degraded by APC\textsuperscript{Cdh1}, APC\textsuperscript{Cdc20}, and either APC\textsuperscript{Cdh1} or APC\textsuperscript{Cdc20}, respectively (Peterson et al. 2000; Jacobs et al. 2001; Zur and Brandeis 2001). In these studies, however, it was not tested whether human CDC6 and Drosophila cyclin A could also be degraded via APC\textsuperscript{Cdc20} and APC\textsuperscript{Cdh1} respectively (Peterson et al. 2000, Jacobs et al. 2001). Taken together, these findings suggest that some proteins require a bipartite KEN
A model for substrate presentation by Cdc20 and Cdh1

An important aspect of our findings is that substrate binding to Cdc20p and Cdh1p is not necessarily sufficient for efficient degradation of that substrate. For example, mutation of the D box or the KEN box of Hsl1p compromised its degradation severely, but still allowed strong binding to Cdh1p or Cdc20p, respectively. Similarly, mutation of the D box stabilizes Clb2p (Amon et al. 1994; Imigier et al. 1995), but had little effect on its binding to Cdc20p or Cdh1p (Fig. 3). Although a KEN box was sufficient for Hsl1p to bind to Cdh1p in vivo, a D box was still needed for what can be termed the “presentation” of Hsl1p to the APC in an orientation that leads to efficient degradation. Our results suggest that both Cdc20p and Cdh1p need to bind both degradation motifs in order to present Hsl1p to the APC properly. A prediction of this model is that Cdc20p and Cdh1p each have two binding sites, one for a D box and one for a KEN box. Do other APC substrates interact with Cdc20p and/or Cdh1p via both a D box and a KEN box? The simple answer would seem to be no as only a minority of APC substrates contain both motifs. Interestingly, the two motifs are usually very close together, being separated by 25 to 53 amino acids (from the start of one motif to the start of the second) in the four characterized proteins with both motifs (human CDC6, human securin, Drosophila cyclin A, and yeast Hsl1p). A large number of potential substrates seem to have both motifs close together (see above). This striking juxtaposition is consistent with simultaneous interaction of the two degradation motifs with two binding pockets on Cdc20p or Cdh1p. In a number of APC substrates, only one motif (usually a D box) has been identified and found necessary for degradation. Perhaps in these cases binding via just one motif can present the substrate properly to the APC. Dual motif binding may be more efficient, but one motif can suffice. Indeed, single mutations in Hsl1p reduced degradation severely, but did not eliminate it. An alternative possibility is that both the D box- and the KEN box-binding regions of Cdc20 and Cdh1 must be engaged for efficient substrate presentation. In such a model, substrates that contain only one discernable degradation motif still must contain sequences that are compatible with the second binding pocket of Cdc20p and/or Cdh1p in order to be presented for ubiquitination by the APC. The main binding energy for the interaction would come from the obvious degradation motif, whereas important positional information could come from a weak interaction provided by a highly degenerate motif. Structural insights will be required to address these issues in further detail.
Materials and methods

Yeast strains and plasmid constructions

All yeast strains are derivatives of W303 J ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100, their relevant genotypes are listed in Table 1. Plasmids are denoted by brackets. Yeast transformations were performed using published methods (Gietz et al. 1995). All PCR products were sequenced to confirm that no extraneous mutations were introduced. Underlined residues in oligonucleotides indicate introduced restriction sites or nucleotide changes, as appropriate. All mutagenesis was performed by Quikchange (Stratagene). Yeast media [YPD and complete minimal (CM)] were prepared as described (Ausubel et al. 1995).

The GAL-HSL1-HA–YEpplac128 and GAL–HSL1mkb–HA–YEpplac128 constructs were described previously (Burton and Solomon 2000). GAL–HSL1mkb–HA–YEpplac128 and GAL–HSL1mkb–HA–YEpplac128 were made from GAL–HSL1–HA–YEpplac128 or GAL–HSL1mkb–HA–YEpplac128, respectively, using oligonucleotides MSO880 (5′-CG-ATC/TCT/CGG/GTG/TCT/ACA/AA/CG/GCA/GCT/GAG/GGC/GCG/TAT/CC A/AA/AA/ATT/GAT/AAG/AAA/CCA/GAA/CTA/TGA/GAA/AGG-3) and MSO881 (5′-CTC/AT/ATT/GTG/TGC/ATG/CCG/GGC/GCC/CAC/ATG/GAT/CCC-3). The nucleotide changes result in the mutagenesis of the KEN box motif [indicates mutated amino acids] K775E776N777XXX781 within Hsl1p to A775A776A777XX781.

GAL–CLB2–HA–YEpplac128 was made by PCR amplification of CLB2 using MSO899 (5′-CCC/GTA/ATG/ATC/TAT/GG/ AAC/ATA/GAA/AAC/GAC-3) and MSO898 (5′-CCC/GTC/GAC/TCT/ATC/GAA/GGT/CAT/GAT/TTA/TAT/ATA/ AA/ATC/CTA/GAA/CTA/CAT/AAG-3), digesting with BamHI and SalI and ligating to GAL–YEpplac128–HA with the same enzymes. The complete GAL–CLB2–HA–YEpplac128 using oligonucleotides MSO991 (5′-GG-ATG/TTA/AGG/ATT/CAA/CTA/CAA,GTT/TTG/GCA/AAC/GT A/A/AA/ATT/GAT/GAT/ATT/AA/AAC/GAC/ATT/GGCC/CTT/GCC/GA/CTA/GAG/GG-3) and MSO992 (5′-CCG/CTT/AAT/ATT/GTA/ATT/GAA/GAA/GGA/GAT/CCC/GAT/GGC/TAT/ATC/GGC/GCG/TTC/TCT/ATG/CTA/CTA/CAA/AA/AAC/GGC/CTT/ATG/ATC/CTA/CTA/GAA/CTA/GAA/CAA-3). The resulting PCR products were cut with NcoI and XhoI and ligated to Bluescript II KS+ (Stratagene) cut with the same enzymes. The GST–CDC20 baculovirus construct was made by subcloning CDC20 into the pVT4 vector (kind gift from Vasiliki Tsakraklides, University of California, San Francisco).

To make maltose-binding protein [MBP]–HSL1 fusions in yeast, the GAL–MBP–YEpplac181 vector was constructed. The malle gene and MCS from pMAL-c2 (New England Biolabs) was amplified using MSO910 (5′-CCC/GAT/ATG/ATC/TGG/ATA/GAA/ATA/ATC/CTA/GAA/AA/ATC/AA/ATA/GAA/AAC/GAC-3) and MSO911 (5′-CCC/AAG/CTT/GCC/TGC/AGC/TGG/ATT/AAA/ATC/GAA/GGA/GGA/ATC/AAA/ATA/ATC/CTC/GAG/GAT/AGG/GGA/CTA/AGG/GAG/GGA/CTA/GAA/GAA/AAA/GCA/ATG/AA/ACT/GAA/GGA/GGA/CTA/AAA/ATA/CTC/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GAA/GA...
| Strain   | Genotype                                      | Reference          |
|----------|-----------------------------------------------|--------------------|
| YJB14    | MATa bar1Δ                                  | Burton and Solomon 2000 |
| YJB15    | MATa bar1Δ:URA3 cdc23-1                      | Burton and Solomon 2000 |
| YJB123   | MATa bar1Δ GAL-HSL1-HA::TRP1                | Burton and Solomon 2000 |
| YJB125   | MATa bar1Δ:URA3 cdc23-1 GAL-HSL1-HA::TRP1   | Burton and Solomon 2000 |
| YJB156   | YJB123 [pEG-KT-CDC20]                       | Burton and Solomon 2000 |
| YJB218   | YJB123 [pEG-KT-CDH1]                        | Burton and Solomon 2000 |
| YJB221   | YJB14 [pEG-KT-CDC20]                        | Burton and Solomon 2000 |
| YJB222   | YJB14 [pEG-KT-CDH1]                         | Burton and Solomon 2000 |
| YJB229   | MATa bar1Δ GAL-HSL1mdb-HA::LEU2             | Burton and Solomon 2000 |
| YJB230   | YJB229 [pEG-KT-CDC20]                       | Burton and Solomon 2000 |
| YJB231   | YJB229 [pEG-KT-CDH1]                        | Burton and Solomon 2000 |
| YJB257   | MATa bar1Δ GAL-HSL1mkb-HA::LEU2             | This study          |
| YJB258   | MATa bar1Δ GAL-HSL1mdb/mkb-HA::LEU2         | This study          |
| YJB259   | YJB257 [pEG-KT-CDC20]                       | This study          |
| YJB260   | YJB258 [pEG-KT-CDC20]                       | This study          |
| YJB261   | YJB257 [pEG-KT-CDH1]                        | This study          |
| YJB262   | YJB258 [pEG-KT-CDH1]                        | This study          |
| YJB265   | MATa bar1Δ:URA3 cdc23-1 GAL-HSL1mdb/mkb-HA::LEU2 | This study          |
| YJB266   | MATa bar1Δ GAL-HSL1-HA::LEU2                | This study          |
| YJB270   | MATa bar1Δ:URA3 cdc23-1 GAL-HSL1mkb-HA::LEU2 | This study          |
| YJB271   | MATa bar1Δ:URA3 cdc23-1 GAL-HSL1mkb-HA::LEU2 | This study          |
| YJB272   | MATa bar1Δ:URA3 cdc23-1 GAL-HSL1mkb-HA::LEU2 | This study          |
| YJB273   | MATa bar1Δ GAL-CBL2-HA::LEU2                | This study          |
| YJB274   | MATa bar1Δ GAL-CBL2mdb-HA::LEU2             | This study          |
| YJB275   | YJB273 [pEG-KT-CDC20]                       | This study          |
| YJB276   | YJB274 [pEG-KT-CDC20]                       | This study          |
| YJB277   | YJB273 [pEG-KT-CDH1]                        | This study          |
| YJB278   | YJB274 [pEG-KT-CDH1]                        | This study          |
| YJB306   | YJB14 [GAL-MBP-HSL1667-872-YEplac181]       | This study          |
| YJB307   | YJB14 [GAL-MBP-HSL1704-872-YEplac181]       | This study          |
| YJB308   | YJB115 [GAL-MBP-HSL1667-872-YEplac181]      | This study          |
| YJB309   | YJB306 [pEG-KT-CDC20]                       | This study          |
| YJB310   | YJB306 [pEG-KT-CDH1]                        | This study          |
| YJB311   | YJB115 [GAL-MBP-HSL1704-872-YEplac181]      | This study          |
| YJB320   | YJB14 [GAL-MBP-HSL1667-837-YEplac181]       | This study          |
| YJB321   | YJB14 [GAL-MBP-HSL1704-872-YEplac181]       | This study          |
| YJB322   | YJB14 [GAL-MBP-HSL1704-872-YEplac181]       | This study          |
| YJB326   | YJB14 [GAL-MBP-HSL1mdb667-872-YEplac181]    | This study          |
| YJB327   | YJB14 [GAL-MBP-HSL1mkb667-872-YEplac181]    | This study          |
| YJB328   | YJB14 [GAL-MBP-HSL1mdb/mkb667-872-YEplac181] | This study          |
| YJB329   | YJB14 [GAL-MBP-HSL1704-837-YEplac181]       | This study          |
| YJB330   | YJB14 [GAL-MBP-HSL1704-837-YEplac181]       | This study          |
| YJB331   | YJB14 [GAL-MBP-HSL1704-837-YEplac181]       | This study          |
| YJB332   | YJB14 [GAL-MBP-HSL1704-837-YEplac181]       | This study          |
| YJB336   | YJB14 [GAL-MBP-HSL1704-837-YEplac181]       | This study          |
| YJB337   | YJB14 [GAL-MBP-HSL1704-837-YEplac181]       | This study          |
| YJB338   | YJB326 [pEG-KT-CDC20]                       | This study          |
| YJB339   | YJB327 [pEG-KT-CDC20]                       | This study          |
| YJB340   | YJB328 [pEG-KT-CDH1]                        | This study          |
| YJB341   | YJB326 [pEG-KT-CDH1]                        | This study          |
| YJB342   | YJB327 [pEG-KT-CDH1]                        | This study          |
| YJB343   | YJB328 [pEG-KT-CDH1]                        | This study          |
| YJB366   | W303a cdc28-13::TRP1 cdh1Δ::LEU2 GAL-HSL1-HA::HIS3 | This study          |
| YJB367   | YJB366 GAL-CDC20-myc::URA3                   | This study          |
| YJB368   | W303a cdc28-13::TRP1 cdh1Δ::LEU2             | This study          |
| YJB377   | YJB368 GAL-HSL1mdb-HA::HIS3                  | This study          |
| YJB378   | YJB377 GAL-CDC20-myc::URA3                   | This study          |
| YJB379   | YJB368 GAL-HSL1mkb-HA::HIS3                  | This study          |
| YJB380   | YJB379 GAL-CDC20-myc::URA3                   | This study          |
37°C for the remainder of the experiment. Levels of protein were monitored by immunoblot analysis as described previously (Burton and Solomon 2000), using either rabbit anti-HA antibodies (50 ng/mL, Santa Cruz) or anti-MBP antibodies (0.36 µg/mL). Quantitative immunoblot analysis was performed on scanned autoradiographs using the program NIH Image 1.62 (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Experimental samples and serial dilutions of Hsl1p-HA from the same autoradiograph were scanned and assigned arbitrary units based on the dilution standard. Plots of wild-type Hsl1p-HA are in arbitrary units, whereas the plots for Hsl1p<sub>mkb</sub>-HA and Hsl1p<sub>mkb</sub>-HA represent the ratios of Hsl1p levels with and without Cdc20-myc expression to adjust for Cdc20p independent degradation of these proteins. Samples for FACS were prepared as described previously (Burton and Solomon 2000).

**Protein extract preparation**

Protein extracts for Hsl1p half-life studies using full-length Hsl1p-HA were prepared as described previously [Burton and Solomon 2000]. For MBP-Hsl1p fusions, extracts were prepared by bead-beating for 4 min in 1× sample buffer (SB; 16.6% SDS, 26% glycerol, 262 mM Tris base, 150 mM DTT) and boiling for 10 min. Samples were spun for 5 min in a microcentrifuge and the supernatants were centrifuged for 10 min at 70,000 rpm at 15°C in a Beckman Optima ultracentrifuge in a TLA-100.2 rotor.

**Common precipitation analysis**

For coexpression of wild-type, mkb, or mkb/mkb forms of full-length Hsl1p-HA with either GST–Cdc20p or GST–Cdh1p, cells were grown overnight in 100 mL of CM-Ura with 1% galactose (4%) in Lysis buffer plus 1 mM EDTA and then resuspended in a 1.7-mL tube in Buffer D plus 1 mM DTT. Beads were pelleted and resuspended in 250 µL of Buffer D plus 1 mM DTT. Glutathione beads were washed three times with 5 mL of wash buffer (10 mM sodium phosphate, pH 8.0, 200 mM NaCl, 1 mM EDTA) and once with 1 mL 1× PBS buffer (10 mM sodium phosphate, pH 7.5, 500 mM NaCl, 1% Triton X-100) and once with 1 mL 1× PBS buffer (10 mM sodium phosphate, pH 7.5, 150 mM NaCl). Beads were transferred to a 1.7-mL tube, pelleted, and resuspended in 200 µL of 1× PBS. Fifty microliters of either bead slurry were then incubated with 1 mL of E. coli extract expressing MBP or MBP–Hsl1p<sub>667-872</sub> isoforms for 2 h at 4°C with rotation. Beads were pelleted, washed three times in 1 mL immunoprecipitation buffer (50 mM potassium HEPES at pH 7.6, 1 mM MgCl<sub>2</sub>, 0.1% Tween-20, 1% glycerol), and then either eluted with 30 µL of Buffer D plus 1 mM DTT and 150 mM imidazole for 30 min at 4°C with rotation for Cdh1p–6xHis beads or resuspended directly in 25 µL of Buffer D plus 0.5 M Tris-HCl at pH 6.8, 357.5 mM sucrose, 162.5 mM β-mercaptoethanol for GST–Cdc20p beads. 6 µL of 5× SB was added to the eluted samples. For Cdh1p–6xHis samples, 25 µL were run on SDS-PAGE for Coomassie staining. 2.5-µL samples were run on SDS-PAGE for immunoblot analysis with anti-MBP antibodies (72 ng/mL). For GST–Cdc20p samples, first 5 µL were processed for immunoblot analysis with anti-MBP antibodies to detect MBP–Hsl1p<sub>667-872</sub> binding. Then the immunoblot was stripped as described previously [Burton and Solomon 2000] and re-probed with anti-GST antibodies to detect GST–Cdc20p present on the glutathione beads.

**In vitro binding assays**

Recombinant MBP or MBP–Hsl1p<sub>667-872</sub> isoforms were produced by induction in E. coli with 0.3 mM IPTG (Sigma) for 2 h at 37°C at an OD<sub>600</sub> of 0.5. Cells were pelleted and then disrupted in Lysis buffer (20 mM Tris-HCl at pH 7.5, 200 mM NaCl) containing protease inhibitors (1 mM PMSF, 10 µg each leupeptin, chymostatin, and pepstatin [Chemicon]) by sonication for three times 1 min on ice with a 5 min rest between pulses (setting 5, Branson sonifier 450). Lysates were clarified by centrifugation at 10,000 rpm for 30 min in an SA600 rotor (Sorvall) at 4°C. Lysates were used either directly or diluted 20-fold for MBP–Hsl1p<sub>667-872</sub> isoforms or 320-fold for MBP for use in binding assays using Cdh1p–6xHis bound to cobalt resin (Talon resin, Clonetech) or GST–Cdc20p bound to glutathione resin (Sigma, see below). For binding assays using in vitro translated [35S]-Cdc20p or [35S]-Cdh1p (see below), lysates were prepared as above except that the Lysis buffer also contained 1 mM EDTA. MBP and MBP–Hsl1p<sub>667-872</sub> isoforms were purified from the E. coli lysates by incubation with amylose resin [New England Biolabs] for 2 h and washed three times with Wash buffer (20 mM Tris-HCl at pH 7.5, 500 mM NaCl, 1 mM EDTA). A final wash was performed in Lysis buffer with 1 mM EDTA.

**CD20– and CDH1–Bluescript plasmids were translated in vitro using the TNT<sup>®</sup>-T7-coupled reticulocyte lysate system (Promega) for 3 h at 30°C. Proteins were labeled using 0.4 µCi/µL [35S]methionine (NEN) in the reaction. 10 µL of the above reaction was incubated with 10 µL of amylose resin containing bound MBP or MBP–Hsl1p<sub>667-872</sub> isoforms (see above) in 1 mL of Lysis buffer plus 1 mM EDTA for 2 h at 4°C with rotation. Beads were then washed three times in 1 mL Wash buffer and samples were run on SDS-PAGE and processed for fluorography.

Cdh1p–6xHis and GST–Cdc20p baculoviruses were used to infect 10<sup>9</sup> Sf9 insect cells at a multiplicity of infection of 5 for 48 h. Infected and uninfected cells were pelleted at 500g for 5 min at 4°C, frozen in liquid nitrogen, and stored at −80°C for future use. Cells were lysed in 15 mL Lysis buffer as described [Fisher et al. 1995]. Lysates were clarified by ultracentrifugation at 40,000 rpm for 30 min at 4°C in a 60Ti rotor (Beckman). Lysates were then incubated with either 500 µL of Talon cobalt resin [50% slurry, Clonetech] or 400 µL glutathione agarose [50% slurry, Sigma] for 1.5 h at 4°C with rotation. Talon beads were washed three times with 5 mL Buffer D (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol at pH 7.8) plus 1 mM DTT and 10 mM imidazole and then resuspended in 1 mL of Buffer D plus 1 mM DTT. Glutathione beads were washed three times with 5 mL of wash buffer (10 mM sodium phosphate at pH 7.5, 500 mM NaCl, 1% Triton X-100) and once with 1 mL 1× PBS buffer [10 mM sodium phosphate at pH 7.5, 150 mM NaCl]. Beads were transferred to a 1.7-mL tube, pelleted, and resuspended in 200 µL of 1× PBS. Fifty microliters of either bead slurry were then incubated with 1 mL of E. coli extract expressing MBP or MBP–Hsl1p<sub>667-872</sub> isoforms for 2 h at 4°C with rotation. Beads were pelleted, washed three times in 1 mL immunoprecipitation buffer (50 mM potassium HEPES at pH 7.6, 1 mM MgCl<sub>2</sub>, 0.1% Tween-20, 10% glycerol), and then either eluted with 30 µL of Buffer D plus 1 mM DTT and 150 mM imidazole for 30 min at 4°C with rotation for Cdh1p–6xHis beads or resuspended directly in 25 µL of 2.5× SB [7.5% SDS, 287.5 mM sucrose, 162.5 mM Tris-HCl at pH 6.8, 357.5 mM β-mercaptoethanol] for GST–Cdc20p beads. 6 µL of 5× SB was added to the eluted samples. For Cdh1p–6xHis samples, 25 µL were run on SDS-PAGE for Coomassie staining. 2.5-µL samples were run on SDS-PAGE for immunoblot analysis with α-MBP antibodies (72 ng/mL). For GST–Cdc20p samples, first 5 µL were processed for immunoblot analysis with anti-MBP antibodies to detect MBP–Hsl1p<sub>667-872</sub> binding. Then the immunoblot was stripped as described previously [Burton and Solomon 2000] and re-probed with anti-GST antibodies to detect GST–Cdc20p present on the glutathione beads.

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