Traversal from G₁ to S-phase in cycling cells of budding yeast is dependent on the destruction of the S-phase cyclin/CDK inhibitor SIC1. Genetic data suggest that SIC1 proteolysis is mediated by the ubiquitin pathway and requires the action of CDC34, CDC4, CDC53, SKP1, and CLN/CDC28. As a first step in defining the functions of the corresponding gene products, we have reconstituted SIC1 multiubiquitination in DEAE-fractionated yeast extract. Multiubiquitination depends on cyclin/CDC28 protein kinase and the CDC34 ubiquitin-conjugating enzyme. Ubiquitin chain formation is abrogated in cdc4Δts mutant extracts and assembly restored by the addition of exogenous CDC4, suggesting a direct role for this protein in SIC1 multiubiquitination. Deletion analysis of SIC1 indicates that the N-terminal 160 residues are both necessary and sufficient to serve as substrate for CDC34-dependent ubiquitination. The complementary C-terminal segment of SIC1 binds to the S-phase cyclin CLB5, indicating a modular structure for SIC1.

INTRODUCTION

The transition from G₁ to S-phase in the budding yeast cell cycle requires several genetic functions, including CDC4, CDC34, CDC53, SKP1, one member of the family of G₁ cyclins (CLN1-CLN3), and one member of the family of S-phase-promoting B-type cyclins (CLB1-CLB6) (Goebel et al., 1988; Schwob et al., 1994; Bai et al., 1996; Mathias et al., 1996; Schneider et al., 1996). An elegant genetic model that accounts for the functions of these genes in the progression from G₁ to S-phase was put forward by Schwob et al. (1994). They observed that at the nonpermissive temperature in cdc34Δts, cdc4Δts, and cdc53Δts mutant cells, the activity of the S-phase-promoting CLB/CDC28 protein kinase is repressed by high levels of the CDK inhibitor SIC1 (Mendenhall, 1993; Nugroho and Mendenhall, 1994). Normally, SIC1 is degraded as wild-type cells negotiate the G₁-S transition (Donovan et al., 1994; Schwob et al., 1994). Cell cycle-regulated destruction of SIC1 fails to occur, however, in cdc4Δts, cdc34Δts, and cdc53Δts mutants. Moreover, accumulation of SIC1 is required for the G₁ arrest phenotype of these mutants, as deletion of SIC1 allows DNA replication to proceed in each of these mutants (Schwob et al., 1994). These observations suggest that CDC34, CDC4, and CDC53 promote the cell cycle-regulated destruction of SIC1, thereby revealing the heretofore cryptic S-phase-promoting protein kinase activity of CLB/CDC28. More recent work has established that SKP1, like CDC4, CDC53, and CDC34, is also required for SIC1 destruction and DNA replication, and the S-phase defect imposed by certain skp1Δts mutant alleles can be rescued by deletion of SIC1 (Bai et al., 1996). Elimination of SIC1 also appears to be a crucial aspect of CLN function during G₁ phase. Clearance of accumulated SIC1 and initiation of DNA replication upon reversal of the cdc34Δts block requires CLN function (Schneider et al., 1996), and cells lacking SIC1 no longer require CLN activity for timely initiation of S-phase (Dirick et al., 1995) or for viability (Schneider et al., 1996; Tyers, 1996).

The proposal that CDC34 promotes SIC1 degradation at the G₁-S transition has considerable appeal, because CDC34 encodes a member of the ubiquitin-conjugating enzyme family (Goebel et al., 1988). Other members of this family of proteins have previously been implicated in the degradation of unstable proteins (Hochstrasser, 1995) via the ubiquitin-proteasome pathway. Ubiquitin, a small highly conserved protein, is first activated at its C terminus by forming a thioester bond with an E1 enzyme. Ubiquitin is subsequently transesterified to a ubiquitin-conjugat-
ing (E2) enzyme like CDC34. Finally, ubiquitin is transferred from the E2 to a lysine residue of the target protein, either directly or with the assistance of a ubiquitin protein ligase (E3). Multiple cycles of ubiquitin transfer result in the assembly of a multiubiquitin chain on the substrate, which, in turn, targets it to the 26S proteasome, where it is degraded. Although the genetic data suggest that SIC1 might be the key S-phase-inhibiting substrate of CDC34, it has not been demonstrated if SIC1 is in fact ubiquitinated and if CDC34 is required for its ubiquitination.

Although the sequence of CDC34 suggests that it may promote degradation of SIC1 via the ubiquitin pathway, it is unclear if CLNs, CDC4, CDC53, and SKP1 contribute directly to this process. For instance, the requirement for CLN/CDC28 kinase may be direct, involving phosphorylation of either SIC1 or the components of the ubiquitination machinery. It is equally possible that CLN/CDC28 promotes the synthesis of G1-specific mRNA transcripts that encode proteins involved in SIC1 turnover (Marini and Reed, 1992; Koch et al., 1996). Furthermore, the sequences of CDC4, CDC53, and SKP1 are not homologous to E1, E2, or E3 components of known ubiquitin-dependent proteolytic pathways, thereby raising the question as to what their exact function is. Both CDC53 and SKP1 are members of novel gene families that are conserved in Saccharomyces cerevisiae, Caenorhabditis elegans, and humans (Zhang et al., 1995; Bai et al., 1996; Kipreos et al., 1996; Mathias et al., 1996). CDC4 contains two recognizable sequence motifs: an SKP1-binding domain termed the “F-box” and eight copies of the WD-40 repeat (Neer et al., 1994; Bai et al., 1996). Genetic data suggest that the products of CDC4, CDC53, CDC34, and SKP1 interact (Bai et al., 1996; Mathias et al., 1996). This prediction has, in part, been confirmed by the assembly of CDC4/SKP1 (Bai et al., 1996) and CDC53/CDC34 (Willems et al., 1996) complexes with recombinant proteins and by the enrichment of CDC4 and CDC53 in affinity-purified preparations of CDC34 (Mathias et al., 1996). Though these interactions are intriguing, it remains unclear whether CDC4, CDC53, and SKP1 participate directly with CDC34 in the ubiquitination of substrates or function in a downstream process such as guiding ubiquitinated substrates to the proteasome.

In the present study, we use DEAE-fractionated yeast extract to demonstrate that SIC1 is efficiently multiubiquitinated in vitro, resulting in at least 85% of the input protein being converted to high molecular weight ubiquitin conjugates. We observe a complete dependence on CLN/CDC28 kinase and CDC34 for high molecular weight ubiquitin conjugate formation. SIC1 accumulates as a phosphoprotein in the absence of CDC34 function and as an unmodified protein in the absence of kinase, suggesting that phosphorylation may precede ubiquitination. In vitro ubiquitin-conjugation assay also revealed a potential ubiquitin ligase-like function for the CDC4 gene product. Deletion analysis of SIC1 was carried out to map sequence elements that target it for destruction. This analysis revealed the existence of a ubiquitination determinant in the N terminus of SIC1 between residues 28 and 37. The C-terminal amino acids 160–284 were dispensable for ubiquitination but were required for binding CLB5/CDC28, the S-phase kinase that is activated upon SIC1 destruction.

**MATERIALS AND METHODS**

**Preparation of Fractionated Yeast Extract**

RJD885 (ura3, leu2, trp1, cdc1::URA3, cdc2::LEU2, cdc3::URA3, cdc4::CEN/ARS-UAS-TRP1, cdc6::CEN/ARS-E2, leu2::GAL-CLN3::LEU2, pep4::TRP1, cdc8::CDC28::HI53, MATa) and RJD983 (ura3, his3, trp1, cdc1::URA3, cdc2::LEU2, cdc3::URA3, cdc4::CEN/ARS-CLN3::LEU2, cdc6::CEN/ARS-E2, cdc8::CDC28::HI53, MATa) cells grown in 2 l of YEP-2% galactose were transferred by centrifugation to 2 l of YEP-2% glucose and incubated for 5.5 h at 24°C to deplete CLN3. Depleted cells were harvested at 4°C, washed once with 200 ml of ice-cold water, and extruded as cell paste into liquid N2. Frozen cells (3.5 g) were ground in a liquid N2-chilled mortar for 10 min, and frozen powder was thawed on ice in 0.5 volumes of B93 (30 mM HEPES, pH 7.2, 100 mM potassium acetate, 1 mM EDTA, 1 mM MgCl₂, 10% glycerol, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) fortified with an additional 200 mM potassium phosphate. Once thawing was complete, extract was incubated on ice for 15 min, centrifuged (15 min, 50,000 rpm, Sorvall RFP100-AT4) to yield a S135 (135,000 × g) fraction, and frozen in liquid N2. S135 (30–45 mg/ml) was thawed; adjusted to 2 mM magnesium acetate, 2 mM CaCl₂, 10 μg/ml RNase, 20 μg/ml DNase, 900 μM/ml hexokinase, and 50 μM glucose; incubated for 30 min at 16°C; and dialyzed once against 1 of CBW (CBW is 25 mM HEPES, pH 7.6, 25 mM NaCl; the dialysis step can be omitted with no detrimental effect). After sedimenting insoluble protein (10 min at 135,000 × g), 90 mg of protein was diluted to 25 ml with CBW and applied to a 5-ml column of DEAE-Sepharose FF. The column was washed consecutively with 2 column volumes of CBW, CBW plus 50 mM NaCl, CBW plus 225 mM NaCl, and CBW plus 500 mM NaCl. Proteins eluting at 250 mM NaCl were concentrated by precipitation with (NH₄)₂SO₄ (80% saturation) or by centrifugal filtration in a Centriprep-10 (Amicon, Beverly, MA), resuspended in 0.75 ml of B93, and dialyzed once against 1 l of B93. Insoluble protein was pelleted by centrifugation (10 min at 135,000 × g), and the supernatant (35 mg of protein in 1.0 ml; 0.25 M DEAE fraction) was frozen in liquid N₂ and stored at −80°C. Fractionation of CDC34 on the DEAE column was monitored by immunoblotting column fractions with anti-CDC34 antiserum (kindly provided by V. Chau, Wayne State Univ.). The 250 mM NaCl eluate was essentially devoid of CDC34, which eluted at 500 mM NaCl. Although the bulk of free ubiquitin flowed through, the 0.25 M DEAE fraction still contained ubiquitin, as determined by immunoblotting.

**Ubiquitination Reactions**

Unless otherwise indicated, ubiquitination reactions contained 100 μg of 0.25 M DEAE fraction, 1 μl of crude or 2 μl of DEAE-fractionated reticulocyte or wheat germ translation reaction containing SIC1, 10 μg of ubiquitin, 100 ng of CDC34, 100 ng of glutathione S-transferase (GST)-CLN2, and an ATP-regenerating system (ARS, Deshaies and Kirschner, 1995). Final concentrations of salts and other compounds were 20 mM HEPES, pH 7.2, 100 mM potassium acetate, 6 mM magnesium acetate, 0.5 mM MgCl₂, 2 mM DTT, 5% glycerol, 0.5 mM PMSF, 0.5 mM EDTA, 5 μg/ml pepstatin, and 5 μg/ml leupeptin. Reactions were incubated at 25°C for the indi-
cated times and processed for SDS-PAGE and fluorography as described (Deshai et al., 1995). Ubiquitin was obtained from Sigma (St. Louis, MO) and GST-CLB2 (Kelloq et al., 1995), GST-CLB2 (Deshai et al. and Kirschner, 1995), CDC34 (Banerjee et al., 1993) and K48R-His6-ubiquitin (Beers and Callis, 1993) were purified from Escherichia coli as described. Methyl-ubiquitin was kindly provided by R. King (Harvard Medical School), and purified UBC4 and RAD6 were kindly provided by V. Chau.

**Preparation of SIC1 Substrates**

SIC1 coding sequences from pMDM169 (Nugroho and Mendenhall, 1994) were excised as a BamHI–PstI fragment and inserted into BamHI plus PstI-digested pGEMI, yielding pRD112. SIC1 produced by transcription/translation of SIC1 coding sequences as described (Deshai et al., 1995) was used for in vitro ubiquitination reactions either in crude form or following batch chromatography on DEAE resin. Equivalent results were obtained by using SIC1 produced in wheat germ or rabbit reticulocyte lysate. Fractionated SIC1 was prepared by mixing 200 μl of a reticulocyte translation reaction with 250 μl of DEAE resin equilibrated with column buffer (CB; 30 mM Tris, pH 8.5, 2 mM DTT, 25 mM NaCl). The resin was washed consecutively with 3 column volumes of CB, CB plus 75 mM NaCl, and CB plus 250 mM NaCl. SIC1 eluting in the 250 mM NaCl wash was exchanged into 20 mM HEPES, pH 7.2, 100 mM potassium acetate, and 2 mM DTT by three cycles of dilution and concentration in a Centricon-10.

To express epitope-tagged SIC1, the coding sequences of SIC1 were excised from pRD112 and cloned into pET11d (Novagen, Madison, WI). A 66-bp double-stranded oligonucleotide encoding a bipartite myc-His epitope was inserted into a NotI site created at the 3' end of SIC1 by PCR mutagenesis. These manipulations result in the addition of the amino acid sequence RPLEEQKLIKEEDLLRHHHHHHHGIP immediately upstream of the C-terminal histidine residue of SIC1. SIC1myc-His2 cloned into pET11d was used as a template for in vitro transcription/translation as described above. SIC1myc-His2 behaved identically to untagged SIC1 in in vitro ubiquitination assays (our unpublished observations).

**SIC1 transcription templates with 5' or 3' terminal deletions were prepared using a PCR method as described (Verma et al., 1997).** The oligonucleotides used to create the indicated deletions are shown below. To generate 5' truncations, the ΔN18, ΔN27, ΔN37, ΔN76, and ΔN158 oligonucleotides were used in conjunction with NotI 3'. The number following Δ refers to the number of codons deleted from the 5' end (e.g., ΔN37 lacks amino acids 1-37): NotI 3', gggttaatacgactcactatag- ggttcatattgctgttcagaggttg; ΔN18, gggttaatacgactcactatagcttagcattgctgcttagtg; ΔN27, gggttaatacgactcactatagcttagcattgctgcttagtg; ΔN37, gggttaatacgactcactatagcttagcattgctgcttagtg; ΔN76, gggttaatacgactcactatagcttagcattgctgcttagtg; and ΔN158, cccctagaggcctgttacagctcactcactcacttgctcagacatgagaaaggg.

The NotI 11-oligonucleotide changes the sequence of the C terminus of SIC1 from DQEH to DQERPH. This change did not affect the ubiquitination of SIC1 (our unpublished results).

To generate 3' truncations, the ΔC24, ΔC74, ΔC98, ΔC125, ΔC155, and ΔC179 oligonucleotides were used in conjunction with WT 5'. The number following Δ refers to the number of codons deleted from the 3' end (e.g., ΔC24 lacks amino acids 261–284): WT 5', ccccaagtatctatgactacaattagcttagctagctacccaccoccc; ΔC24, ggcctgaaccttacactactacactacttcctctcctc; ΔC74, ggcctgaaccttacactactacactacttcctctcctc; ΔC98, ggcctgaaccttacactactacactacttcctctcctc; ΔC125, ggcctgaaccttacactactacactacttcctctcctc; and ΔC155, ggcctgaaccttacactactacactacttcctctcctc.

**Alkaline Phosphatase Treatment of SIC1**

A 10-μl in vitro ubiquitination reaction lacking CDC34 and containing 2 μl of reticulocyte-translated SIC1myc-His2 was diluted with 190 μl of IPB (IPB; 25 mM Tris, pH 7.5, 0.1% Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM PMSF, 2.5 μg/ml pepstatin, 2.5 μg/ml leupeptin), supplemented with 1 μl of 9E10 ascites fluid (Evan et al., 1985) and incubated for 45 min at 4°C. Immune complexes retrieved by incubation with anti-mouse IgG Sepharose beads (Sigma; 15 μl, packed volume) were washed twice with IPB and twice with 50 mM Tris, pH 8.8. Washed beads were resuspended in 50 mM Tris, pH 8.8, treated with 20 U alkaline phosphatase in the presence or absence of 100 mM sodium phosphate, pH 8.8, at 37°C for 15 min, and processed for SDS-PAGE and autoradiography (Deshai et al., 1995).

**Baculovirus Expression of CDC4 in Insect Cells**

To generate a recombinant baculovirus expressing CDC4, the full-length CDC4 gene contained in the plasmid pCD4–7 (kindly provided by B. Jensen, University of Washington, Seattle, WA) was amplified by PCR using oligonucleotides RDO72 (GAAGCGTACGATCCATGCTGGTCGCTTTAGAC) and RDO73 (GAAGCGTGCAGCTCGATATTATATATGCTTCT). The resulting product was digested with SpeI plus SacI and ligated into pRS306 that had been digested with Xhol plus Sall. The PCR-derived part of CDC4 was checked by DNA sequencing. The recombinant plasmid was digested with Xhol, treated with Klenow fragment, digested with Eagl, and ligated into the baculovirus transfer vector pVL1392 that had been digested with Eagl and Smal. The resulting transfer vector was mixed with BaculoGold viral DNA (Pharmingen, San Diego, CA) and transfected into S9 insect cells. Plaques expressing CDC4 as assayed by Western blot analysis were purified and amplified in S9 cells.

Cell lysates were prepared by infecting 100-mm dishes containing 106 S9 cells with virus at a multiplicity of infection of 10. After 43 h, cells were harvested, washed twice with Tris-buffered saline, pH 7.5, resuspended in 1 ml of ice-cold BVL buffer (20 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 5 mM MgCl2, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin), and sonicated briefly to ensure lysis. After pelleting cell debris (15 min at 13,000 × g), the lysate was dialyzed twice for 2 h against 1 l of buffer B93. The dialysate was centrifuged (10 min at 13,000 × g) and stored at −80°C. Cell lysates contained approximately 4 mg/ml total protein, approximately 0.25% of which was CDC4.

**Binding of SIC1 to CLB5**

Congenic strains K3819 (RJD916; ade2–1, trpl–1, can1–100, leu2–3,–112, his3–11,–15, ura3–1,–110; ADH1-CLB5; UR3, MATa) and W303 (RJD360; can1–100, leu2–3,–112, his3–11,–15, trpl–1, ura3–1, ade2–1, MATa) were grown in YEPD medium, and cell extracts (5 mg protein/ml) were prepared by agitation with glass beads in cell lysis buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM EGTA, 2 mM sodium vanadate, 60 mM β-glycerolphosphate, 2 mM DTT, 10 mM NaF, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin). Each binding reaction contained 200 μl (1 mg) of yeast extract, 1 μl of anti-HA ascites fluid, and 2 μl of "normalized" wheat germ translation product (translation products were adjusted to similar concentrations by dilution with an unprogrammed wheat germ translation reaction). Following a 60-min incubation on ice, immune complexes were collected on a protein A matrix, washed three times with cell lysis buffer, and analyzed by SDS-PAGE and fluorography.

**RESULTS**

**Reconstitution of SIC1 Ubiquitination in Fractionated Yeast Extract**

A substantial body of genetic and biochemical data indicate that the specific and regulated assembly of
Figure 1. SIC1 is mult ubiquitinated in vitro. (A) Time-dependent conversion of SIC1 to higher molecular mass forms by yeast extract. Extract from cells arrested in G1 due to CLN depletion was fractionated by chromatography on DEAE resin and supplemented with ubiquitin (Ub), CDC34, GST-CLN2, and ARS. 35S-labeled SIC1 synthesized in reticulocyte lysate and fractionated by batch chromatography on DEAE was added and the reaction incubated at 25°C for the indicated lengths of time. Reactions were terminated and samples were resolved by SDS-PAGE and processed for autoradiography as described (Deshaies et al., 1995). (B) Conversion of SIC1 to high molecular mass forms requires ubiquitin. Enriched SIC1 translation product was incubated 15 min in the absence (lane 1) or presence (lanes 2–8) of 0.25 M DEAE fraction containing CDC34, GST-CLN2, and ARS. The reactions displayed in lanes 2 (10 μg), 4 (2.5 μg), 5 (5.0 μg), and 6 (10 μg) were supplemented with the indicated amounts of ubiquitin. Ubiquitin was omitted from the reaction shown in lane 3 and substituted by methyl-ubiquitin (15 μg) or the mutant K48R-ubiquitin (15 μg) in lanes 7 and 8, as indicated.

multiubiquitin chains on substrate proteins targets them for exhaustive proteolysis by the 26S proteasome (Hochstrasser, 1995). To investigate the degradation of SIC1, we thus sought to establish a fractionated in vitro system that supported efficient CDC34-dependent mult ubiquitination. CLN2 was previously shown to be ubiquitinated in a CDC34-dependent manner in crude whole-cell yeast extract (Deshaies et al., 1995). Equivalent ubiquitination-competent crude extracts of CLN-depleted G1-arrested yeast cells were applied to DEAE resin, and bound proteins eluted by 250 mM NaCl were pooled and concentrated as described in MATERIALS AND METHODS. The resulting G1 cyclin, ubiquitin, and CDC34-depleted 0.25 M DEAE fraction sustained—upon addition of an ARS, ubiquitin, CDC34, and in vitro-translated CLN2—both the assembly of active CLN2/CDC28 complexes and the CDC34-dependent ubiquitination of CLN2 (Verma et al., 1997; our unpublished observations).

The 0.25 M DEAE fraction was then tested for its ability to ubiquitinate SIC1. 35S-labeled SIC1 translation product was prepared by in vitro transcription followed by translation of SIC1 mRNA in rabbit reticulocyte lysate, yielding a major 40-kDa product and smaller amounts of an ~38-kDa species (Figure 1A, lane 6). Upon incubation for increasing amounts of time with the 0.25 M DEAE fraction supplemented with ARS, ubiquitin, CDC34, and GST-CLN2, SIC1 was rapidly and efficiently converted to a ladder of higher molecular weight forms (Figure 1A, lanes 1–5). These modifications were catalyzed by activities present in yeast cell extract, since SIC1 was not modified if 0.25 M DEAE fraction was omitted from the reaction (Figure 1B, lane 1). Presumably, E1 and potential E3s needed for SIC1 ubiquitination cofractionated in the 0.25 M DEAE cut.

Two pieces of evidence indicate that the high molecular mass forms of SIC1 produced in yeast cell extract arise from mult ubiquitination: the conversion of SIC1 to high molecular mass forms was diminished either if ubiquitin was omitted from the reaction (Figure 1B, lane 3; immunoblotting indicated that the 0.25 M DEAE fraction was contaminated with low levels of ubiquitin) or if methyl-ubiquitin or K48R-ubiquitin was added to the reaction (Figure 1B, lanes 7 and 8). Methylation of the ε-amino group of lysines or mutation of lysine 48 in ubiquitin (Ub) blocks mult ubiquitin chain formation by preventing Ub-Ub ligation (Hershko and Heller, 1985; Chau et al., 1989). In sum, the data in Figure 1 demonstrate that mult ubiquitination of SIC1 in fractionated yeast extract was rapid, efficient, and highly processive. Indeed, the CDC34-dependent accumulation of mult ubiquitinated SIC1 was severalfold more rapid and 5- to 10-fold more efficient than what is observed using CLN2 as a substrate (Deshaies et al., 1995; Verma et al., 1997). A similar spectrum of high molecular weight ubiquitini-
Ubiquitination of SIC1 Requires CDC34 and CDC4

The disappearance of SIC1 at the G1-S transition in vivo requires the activity of the ubiquitin conjugating enzyme encoded by CDC34 (Schwob et al., 1994). Presumably due to its highly acidic C-terminal tail (Goebl et al., 1988), CDC34 bound avidly to DEAE resin and was eluted only at NaCl concentrations exceeding ~0.35 M, as judged by immunoblotting (our unpublished data). Thus, to test whether the CDC34 ubiquitin conjugating enzyme participates directly in the multiubiquitination of SIC1, in vitro incubations were performed with 0.25 M DEAE fraction as described above except that CDC34 was omitted. As shown in Figure 2A, no ubiquitination of SIC1 occurred in the absence of CDC34 (lane 3). Instead, SIC1 accumulated in a form that migrated with reduced mobility (by approximately 2 kDa) as compared with the translation product (lane 1). We show later that this 2-kDa shifted form arose from phosphorylation (see below). The requirement for CDC34 was specific: UBC4 and RAD6 ubiquitin-conjugating enzymes failed to catalyze SIC1 ubiquitination (lanes 4 and 5). Titration experiments indicated that CDC34 restored efficient ubiquitination at concentrations as low as 30 nM whereas UBC4 and RAD6 failed to restore ubiquitination even at 30-fold higher levels, concentrations at which CDC34 was saturating but not inhibitory (lanes 4 and 5 and our unpublished results). By comparison, measurements of [35S]methionine incorporation indicated that ubiquitination reactions contained 1–5 nM SIC1.

To test whether CDC4 is also required for the multiubiquitination of SIC1 observed in DEAE-fractionated extract, we prepared 0.25 M DEAE fraction from CLN-depleted G1-arrested cdc4ts mutant cells. In contrast to the results obtained with a 0.25 M DEAE fraction prepared from wild-type cells (Figure 2B, lane 2).

Figure 2. CDC34 and CDC4 are required for SIC1 multiubiquitination. (A) Ubiquitination of SIC1 requires CDC34. Enriched 35S-labeled SIC1 translation product was incubated 15 min at 25°C in the absence (lane 1) or presence (lanes 2–5) of 0.25 M DEAE fraction supplemented with ubiquitin (Ub), ARS, and GST-CLN2. The reaction displayed in lane 2 contained 0.5 μg of CDC34. CDC34 was omitted from the reaction shown in lane 3 and substituted by 1.0 μg of UBC4 or 1.0 μg of RAD6 in the reactions shown in lanes 4 and 5, respectively. (B) Ubiquitination of SIC1 requires CDC4. 35S-labeled SIC1 translation product (lane 1) was incubated with CDC34, ubiquitin, ARS, GST-CLN2, and 0.25M DEAE-fractionated extract (xtr) from wild-type CDC4+ (lane 2) or cdc4ts (lanes 3–9) cells; yeast extract was absent in lanes 1, 10, and 11. Lanes 4 and 5 contain cell lysate from uninfected Sf9 cells; lanes 6 and 7 contain lysate from Xenopus CDC2-expressing Sf9 cells; and lanes 8–11 contain lysate from CDC4-expressing Sf9 cells. Lanes 5, 7, and 9 contain a 1:3 dilution of the corresponding Sf9 cell lysate from lanes 4, 6, and 8, respectively. Reactions were carried out and processed as described in Figure 1A.
2), the 0.25 M DEAE fraction isolated from cdc4ts cells failed to sustain the assembly of high molecular mass SIC1–ubiquitin conjugates (lane 3). This ubiquitination defect was specific, since addition of SF9 insect cell lysates containing CDC4 expressed from a recombinant baculovirus restored SIC1 ubiquitination (lanes 8 and 9), whereas SF9 lysates from uninfected cells (lanes 4 and 5) or from cells infected with a Xenopus CDC2-expressing baculovirus (lanes 6 and 7) had no effect. Estimates of the CDC4 concentration in SF9 lysates (via immunoblotting) indicated that efficient rescue of cdc4ts extract was achieved with 1–10 nM CDC4 (our unpublished observations).

**Phosphorylation and Ubiquitination of SIC1 Require CLN2**

Genetic studies indicate that continuous CLN expression is needed for SIC1 turnover in cells released from a cdc34ts conditional block (Schneider et al., 1996). Additionally, cells with deleted CLNs can be rescued by deleting SIC1, implying that CLN-dependent inactivation of SIC1 is rate-limiting for entry into S-phase and cell growth (Dirick et al., 1995; Schneider et al., 1996; Tyers, 1996). To test whether CLN/CDC28 protein kinase activity is directly required for mult ubiquitination of SIC1, we assembled ubiquitination reactions lacking exogenously added GST-CLN2. Since the 0.25 M DEAE fraction was prepared from G2-arrested cyclin-depleted cells, it contained little or no CDC28-associated protein kinase activity (Deshaies et al., 1995; Deshaies and Kirschner, 1995). Upon addition of GST-CLN2 or GST-CLB2, however, the 0.25 M DEAE fraction generated CDC34-associated protein kinase activity, as was reported for unfractionated extracts (Deshaies and Kirschner, 1995; unpublished observations).

Whereas SIC1 was efficiently ubiquitinated in 0.25 M DEAE fraction supplemented with GST-CLN2 or GST-CLB2 (Figure 3A, lanes 2 and 4), neither ubiquitination nor the 2-kDa shift in molecular mass were observed when cyclin was omitted from the reaction (Figure 3A, lane 3). Similarly, SIC1 ubiquitination and the 2-kDa shift in molecular mass were significantly reduced in reactions performed with extract prepared from G2-arrested cdc28ts cells (our unpublished results). The cyclin-dependent 2-kDa shift in molecular mass was due to phosphorylation of SIC1, since the “shifted” 42-kDa form of epitope-tagged SIC1 (SIC1mycHis6) that accumulated in reactions containing GST-CLN2 but lacking CDC34 (Figure 3B, lane 3) was converted to the 40-kDa form upon incubation with alkaline phosphatase (Figure 3B, lane 5). This 2-kDa shift in molecular weight most likely arises from direct phosphorylation of SIC1 by CDC28, since CLN2/CDC28 complexes immunosolated from yeast cells can phosphorylate purified bacterially expressed SIC1 (Schwob et al., 1994; our unpublished observations). The above data are consistent with earlier studies showing accumulation of SIC1 as a phosphorylated protein in cdc34ts cells as compared with cdc28ts cells.

![Figure 3](image-url)
Figure 4. The N-terminal domain of SIC1 is both necessary and sufficient for ubiquitination. $^{35}$S-labeled SIC1 truncation mutants generated by transcription/translation of mutant PCR-derived templates were incubated under standard reaction conditions with 0.25 M DEAE fraction supplemented with ARS, ubiquitin, CDC34, and GST-CLN2. At the indicated time points, samples were removed and ubiquitination was assessed by SDS-PAGE and fluorography. (A) Ubiquitination of N-terminal truncation mutant lacking amino acids 1–37. (B) Ubiquitination of C-terminal truncation mutants.

The number following AC refers to the number of codons deleted from the 3' end (e.g., AC24 lacks the C-terminal amino acids 261–284). In lanes 19–21 (exposed to film for a longer period of time), ubiquitination reactions were assembled fully supplemented as described above (lane 20) or in the absence of yeast extract (lane 19) or GST-CLN2 (lane 21).

(Donovan et al., 1994; Schwob et al., 1994; Schneider et al., 1996).

The N-Terminal Domain of SIC1 Contains a Ubiquitination Determinant

To begin an analysis of how the CDC34 pathway selects SIC1 as a substrate for ubiquitination, we constructed a series of 5' and 3' truncations of SIC1 by PCR. PCR templates were transcribed and translated in vitro, and the amount of protein translated from each template was estimated by quantitation of SDS-polyacrylamide gels. Equivalent amounts of wild-type and mutant proteins were mixed with fully supplemented 0.25 M DEAE fraction and incubated under standard reaction conditions. Whereas wild-type SIC1 yielded a characteristic pattern of ubiquitin conjugates (Figure 4A, lane 2), a mutant lacking the first 37 amino acids was a poor substrate (lane 6), suggesting that the N terminus of SIC1 contains a determinant that is essential for its recognition by the CDC34 pathway. Since a truncation mutant lacking the first 27 amino acids was only mildly defective for ubiquitination (our unpublished results), the determinant could be narrowed down to residues 28 through 37.

A complementary set of C-terminal deletions was generated to test whether the N-terminal domain of SIC1 was sufficient to serve as a substrate for CDC34-dependent ubiquitination. Deletion of as many as 125 residues from the C terminus of SIC1 had only a modest effect on ubiquitination (ΔC125 mutant; Figure 4B, lanes 10–12), which remained dependent upon CDC34 and CLN2 (our unpublished data). In contrast, removal of 30 more amino acids compromised the ability of SIC1 to serve as a substrate for ubiquitination (ΔC155, lanes 13–15). Nevertheless, a deletion mutant retaining only the first 105 amino acids of SIC1 was still ubiquitinated in vitro (ΔC179, lanes 16–18) in a reaction that remained dependent on the addition of GST-CLN2 (lanes 20 and 21). The diminished ubiquitination of the ΔC155 and ΔC179 mutants correlated with a reduced phosphorylation-dependent mobility shift. Since the 30 amino acid segment between amino acids 130 and 160 does not contain any consensus sites for CDC28-dependent phosphorylation, it is unclear if this region contains a binding site required for interaction with CLN2/CDC28 or is required for proper folding of the N-terminal domain.

Ubiquitination of SIC1 Does Not Depend upon Its Association with CLB5/CDC28

The same truncation mutants that were used to map ubiquitination determinants in SIC1 were also tested for their ability to bind to CLB5/CDC28 complexes. Wheat germ lysates containing in vitro-translated [35S]methionine-labeled wild-type or mutant SIC1 proteins were mixed with either control yeast extract or yeast extract containing hemagglutinin-tagged CLB5 (CLB5HA). CLB5HA and associated proteins were then retrieved by immunoprecipitation with an anti-hemagglutinin monoclonal antibody (anti-HA), and the immune complexes were evaluated by SDS-PAGE and fluorography (Figure 5). Whereas SIC1 mutants lacking as few as 37 residues from the N terminus were poor substrates for the CDC34 pathway (Figure 4A, lane 6), deletion of up to 160 residues from the N terminus had no effect on the interaction between SIC1 and CLB5HA (Figure 5 bottom, lanes 7–9). In contrast, deletion of 24 residues from the C termi-
**Factors Required for SIC1 Ubiquitination**

Previously, it had been shown in vivo that cdc4<sup>ts</sup>, cdc34<sup>ts</sup>, cdc53<sup>ts</sup>, skp<sup>ts</sup>, and CLN-deficient mutants fail to initiate both S-phase and SIC1 proteolysis (Goebi et al., 1988; Schwob et al., 1994; Bai et al., 1996; Mathias et al., 1996; Schneider et al., 1996). Given that deletion of SIC1 relieves the S-phase block in these mutants, it was proposed that CDC34, CDC4, CDC53, SKP1, and CLNs promote the initiation of DNA replication by specifying the elimination of SIC1 (Schwob et al., 1994). The results presented herein provide strong support for this model and suggest that CDC4 and CDC34 promote SIC1 degradation by directly catalyzing the assembly of multiubiquitin chains upon SIC1. The results also prompt the question of whether CDC53 and SKP1 are required for SIC1 multiubiquitination. Although cdc53<sup>ts</sup> mutant extracts were defective for SIC1 ubiquitination, we were unable to rescue them with S9 insect cell lysates containing CDC53 expressed from a recombinant baculovirus. We did not test skp1 mutant alleles since their SIC1 proteolysis defect appears to be rather leaky (Bai et al., 1996). We have shown, however, that recombinant SKP1 and CDC53 are required for CDC4/CDC34/CLN-dependent ubiquitination of SIC1 in crude insect cell lysates (Feldman et al., unpublished results).

**How Does CDC4 Promote the CDC34-dependent Ubiquitination of SIC1?**

The sequence of CDC4 provides little clue as to how it promotes SIC1 multiubiquitination. CDC4 contains two recognizable motifs: an SKP1-binding domain known as an F-box and eight copies of the WD-40 repeat (Neer et al., 1994; Bai et al., 1996). The crystal structure of the β-subunit of transducin reveals that its seven WD-40 repeats fold into a β-propeller structure with seven blades evenly distributed about a central axis. This structure provides a platform for interaction of the β-subunit with the α- and γ-subunits of transducin (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996). Likewise, the WD-40 repeats of CDC4 may fold into a structure that promotes interactions between CDC4 and other ubiquitination factors or substrates.

Multibuquitination of substrates that are degraded by the ubiquitin-dependent proteolytic pathway often requires E3 enzymes or accessory factors. Surprisingly, the two most well-studied E3s UBR1 (Bartel et al., 1990) and E6-AP (Huibregtse et al., 1993) are not homologous to each other or to CDC4. Furthermore, CDC4 lacks the conserved cysteine-containing motif that has been implicated in the ubiquitination-promoting activity of E6-AP and its homologs (Scheffner et al., 1995). An understanding of the mechanism of action of CDC4 in the ubiquitination of SIC1 awaits a detailed dissection of this process.
How Do CLNs Promote the Ubiquitination of SIC1?
The accumulation of SIC1 is under cell cycle control. SIC1 begins to accumulate in telophase as the decline in CLB/CDC28 protein kinase activity enables an increase in SIC1 transcription due to nuclear translocation of the transcription factor SWI5 (Chau et al., 1989). During early (pre-START) G1 phase, newly synthesized SIC1 is stable and can persist for long periods of time in cells arrested prior to START by mutations or nutrient starvation (Mendenhall et al., 1987; Schwob et al., 1994). Upon traversal of START, active CLN/CDC28 complexes are assembled and SIC1 is degraded shortly thereafter (Schneider et al., 1996). Degradation of SIC1 depends upon CLN/CDC28 activity, as SIC1 persists in cdc28ts and CLN-depleted cells (Schwob et al., 1994; Schneider et al., 1996). Whereas many of the processes downstream of START may be activated by the CLN/CDC28-dependent synthesis of new proteins, the results presented herein argue that CLN/CDC28 enables SIC1 destruction by a direct mechanism. CLN/CDC28 may promote SIC1 ubiquitination and degradation by directly phosphorylating SIC1 and rendering it susceptible to ubiquitination by a constitutively active CDC34 pathway, by activating an essential component of the CDC34 ubiquitination pathway, by inactivating an inhibitor of the CDC34 pathway, or by serving to bridge the assembly of SIC1 with ubiquitination factors such as CDC53 (Willems et al., 1996). Experiments designed to distinguish conclusively between these four possibilities are currently underway.

How Does the G1-S Transition Work?
The observations reported herein raise several questions about the mechanism of the G1-S transition in vivo. If CLN/CDC28 activity directly triggers SIC1 ubiquitination and subsequent proteolysis, does the rate of SIC1 destruction (and, conversely, the level of CLB5/CDC28 activity) mirror the rate of CLN accumulation or are there mechanisms that restrain SIC1 proteolysis until CLN/CDC28 activity exceeds a threshold? Since sic1Δ cells enter S-phase precociously (Schneider et al., 1996), it seems plausible that there exists a window of time in G1 phase between the accumulation of SBF- and MBF-promoted gene products, such as CLN1,2 and CLB5, and the degradation of SIC1 (Dirick et al., 1995). If this is so, then how might SIC1 destruction be restrained in cells with active CLN/CDC28? Perhaps the ability of CLN/CDC28 to activate SIC1 destruction is opposed by a potent phosphatase; this possibility is supported by the observation that degradation of SIC1 upon reversal of the cdc28ts mutant block requires the continuous synthesis of CLN protein (Schneider et al., 1996).

One factor that may contribute to the sharpness of the G1-S transition in vivo is the potential for positive feedback in the degradation of SIC1. Since mult ubiquitination of SIC1 in vitro can be triggered by CLB2/CDC28 (Figure 3A, lane 4) and CLB5/CDC28 (Feldman, unpublished data), an initial burst of SIC1 destruction triggered by CLN/CDC28 would lead to the emergence of a small pool of liberated CLB5/CDC28 complexes, which in turn could positively feed back to accelerate the rate of SIC1 ubiquitination and degradation. A detailed understanding of the relationship between CLN/CDC28 activity and the dynamics of the G1-S transition in vivo would be facilitated by the development of methods to monitor in real time the degradation of SIC1 in single cells.

Modular Organization of SIC1
The N-terminal ubiquitination domain of SIC1 contains a high density of potential CDC28 phosphorylation sites—there are seven (S/T)P dipeptides within the first 81 amino acids—and an N-terminal fragment containing the first 105 amino acids of SIC1 is a substrate for CLN2/CDC28-dependent phosphorylation. Aside from potential phosphorylation sites, what other determinants within the N-terminal domain of SIC1 might target it for CDC34-dependent ubiquitination? SIC1 has been reported to contain PEST sequences located at amino acids 37-49 (PEST score -2.0), 115-141 (PEST score -2.1), and 198-212 (PEST score +2.0) (Nugroho and Mendenhall, 1994; Rechsteiner and Rogers, 1996). PEST sequences are compositional elements that are rich in proline, glutamic acid, serine, and threonine; they are often found in unstable proteins. According to Rechsteiner and Rogers (1996), however, only segments with scores above zero are normally considered as possible PEST regions. The three PEST elements within SIC1 are apparently not sufficient to direct its ubiquitination, since SIC1 ΔN37 is poorly ubiquitinated. Moreover, the SIC1 ΔC125 C-terminal truncation mutant, which lacks the only one of the three PEST regions with a positive score, is nevertheless a good substrate for CDC34-dependent ubiquitination. Since SIC1 ΔN27 is ubiquitinated with moderate efficiency (our unpublished observations) and SIC1 ΔN37 is a poor substrate, residues 28-37 provide an essential ubiquitination determinant. The nature of the signal contained in this segment is unclear at present. It is unlikely to be sufficient as a destruction signal however, since we have created mutations elsewhere in the N-terminal domain of SIC1 that eliminate ubiquitination (our unpublished results). A complete delineation of the sequence elements that specify SIC1 ubiquitination will require a more extensive mutagenic analysis of the N-terminal domain of SIC1. Deletion analysis revealed that a C-terminal 126 amino acid segment of SIC1 is sufficient to bind CLB5/CDC28 complexes and that the C-terminal 24 amino
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References


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