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SIC1 Is Ubiquitinated In Vitro by a Pathway that Requires CDC4, CDC34, and Cyclin/CDK Activities

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Traversal from G_1 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 $cdc4^{ts}$ 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 G_1 to S-phase in the budding yeast cell cycle requires several genetic functions, including *CDC4*, *CDC34*, *CDC53*, *SKP1*, one member of the family of G_1 cyclins (*CLN1-CLN3*), and one member of the family of S-phase-promoting B-type cyclins (*CLB1-CLB6*) (Goebl *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 G_1 to S-phase was put forward by Schwob *et al.* (1994). They observed that at the nonpermissive temperature in $cdc34^{ts}$, $cdc4^{ts}$, and $cdc53^{ts}$ 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 G_1–S transition (Donovan *et al.*, 1994; Schwob *et al.*, 1994). Cell cycle-regulated destruction of SIC1 fails to occur, however, in $cdc4^{ts}$, $cdc34^{ts}$, and $cdc53^{ts}$ mutants. Moreover, accumulation of SIC1 is required for the G_1 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 $skp1^{ts}$ 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 G_1 phase. Clearance of accumulated SIC1 and initiation of DNA replication upon reversal of the $cdc34^{ts}$ 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 G_1–S transition has considerable appeal, because *CDC34* encodes a member of the ubiquitin-conjugating enzyme family (Goebl *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-

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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 G_1-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. The in vitro ubiquitination 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 dispensible 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, cln1::URA3, cln2::LEU2, cln3::URA3, leu2::GAL-CLN3::LEU2, pep4::TRP1, cdc28::CDC28^{HA}::HIS3, MATα*) and RJD893 (*ura3, his3, trp1, cln1::URA3, cln2::LEU2, cln3::URA3, leu2::GAL-CLN3::LEU2, cdc4, pep4::TRP1, cdc28::CDC28,*itusp;HA.*::HIS3, MAT***a**) 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 N_2. Frozen cells (3.5 g) were ground in a liquid N_2-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 $MgCl_2$, 10% glycerol, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)] fortified with an additional 200 mM potassium acetate. Once thawing was complete, extract was incubated on ice for 15 min, centrifuged (15 min, 50,000 rpm, Sorvall RP100-AT4) to yield an S135 (135,000 × *g*) fraction, and frozen in liquid N_2. S135 (30–45 mg/ml) was thawed; adjusted to 2 mM magnesium acetate, 2 mM $CaCl_2$, 10 μg/ml RNase, 20 μg/ml DNase, 900 mU/ml hexokinase, and 50 mM glucose; incubated for 30 min at 16°C; and dialyzed once against 1 l of CWB (CWB 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*), 96 mg of protein was diluted to 25 ml with CWB and applied to a 5-ml column of DEAE-Sepharose FF. The column was washed consecutively with 2 column volumes of CWB, CWB plus 50 mM NaCl, CWB plus 225 mM NaCl, and CWB plus 500 mM NaCl. Proteins eluting at 250 mM NaCl were concentrated by precipitation with $(NH_4)_2SO_4$ (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 N_2 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 glutathionine *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 $MgCl_2$, 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 (Deshaies *et al.*, 1995). Ubiquitin was obtained from Sigma (St. Louis, MO), and GST-CLB2 (Kellogg *et al.*, 1995), GST-CLN2 (Deshaies 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 *Bam*HI–*Pst*I fragment and inserted into *Bam*HI plus *Pst*I-digested pGEM1, yielding pRD112. SIC1 produced by transcription/translation of *SIC1* coding sequences as described (Deshaies *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-His6 epitope was inserted into a *Not*I site created at the 3′ end of *SIC1* by PCR mutagenesis. These manipulations result in the addition of the amino acid sequence RPLEEQKLISEEDLL-RHHHHHHGP immediately upstream of the C-terminal histidine residue of SIC1. $SIC1^{myc\text{-}His6}$ cloned into pET11d was used as a template for in vitro transcription/translation as described above. $SIC1^{myc\text{-}His6}$ 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 *Not*I 3′. The number following Δ refers to the number of codons deleted from the 5′ end (e.g., ΔN37 lacks amino acids 1–37): *Not*I 3′, gcgggatcctcaatgcggccgctcttgatccctagattg; ΔN18, gggttaatacgactcactatagatccatgagtggcaatactagttctagtg; ΔN27, gggttaatacgactcactataagatctcatatgcaaggtcaaaagacccc; ΔN37, gggttaatacgactcactataggatccatgtcacagaacctagtccctgtc; ΔN76, gggttaatacgactcactataagatctcatatgcctcaacgctcgccgtttcc; and ΔN158, cccttaatacgactcactataggatccatatggatcagacacatagaaagaag.

The *Not*I 3′ 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′, cccgaattcttaatacgactcactataagatctcatatgactccttccacccc; ΔC24, gggatcctcatttctctaccacctctcccttc; ΔC74, ggggatcctcaattgatgatgatgtctcctcg; ΔC98, ggggatcctcaatttttgccaattcaaatg; ΔC125, ggggatcctcaatcaaactcatctctttcaagtg; ΔC155, ggggatcctcattctgctgcatctatatccac; and ΔC179, ggggatccctactcttcccttactgttcc.

Alkaline Phosphatase Treatment of SIC1

A 10-μl in vitro ubiquitination reaction lacking CDC34 and containing 2 μl of reticulocyte-translated $SIC1^{myc\text{-}His6}$ 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 hydrochlorine (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 IgG1 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 (Deshaies *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 pCDC4–73 (kindly provided by B. Jensen, University of Washington, Seattle, WA) was amplified by PCR using oligonucleotides RDO 72 (GAACGACTAG-TACCATGGGGTCGTTTCCCTTAGC) and RDO 73 (GAACGGTC-GACTCATGGTATTATAGTTGTCC). The resulting product was digested with *Spe*I plus *Sal*I and ligated into pRS306 that had been digested with *Xba*I plus *Sal*I. The PCR-derived part of *CDC4* was checked by DNA sequencing. The recombinant plasmid was digested with *Xho*I, treated with Klenow fragment, digested with *Eag*I, and ligated into the baculovirus transfer vector pVL1392 that had been digested with *Eag*I and *Sma*I. The resulting transfer vector was mixed with BaculoGold viral DNA (PharMingen, San Diego, CA) and transfected into Sf9 insect cells. Plaques expressing CDC4 as assayed by Western blot analysis were purified and amplified in Sf9 cells.

Cell lysates were prepared by infecting 100-mm dishes containing 10^7 Sf9 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 $MgCl_2$, 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 clarified by centrifugation (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, trp1–1, can1–100, leu2–3,-112, his3–11,-15, ura3::ADH-HA-CLB5::URA3, MAT***a**) and W303 (RJD360; *can1–100, leu2–3,-112, his3–11,-15, trp1–1, ura3–1, ade2–1, MAT***a**) 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-glycerophosphate, 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

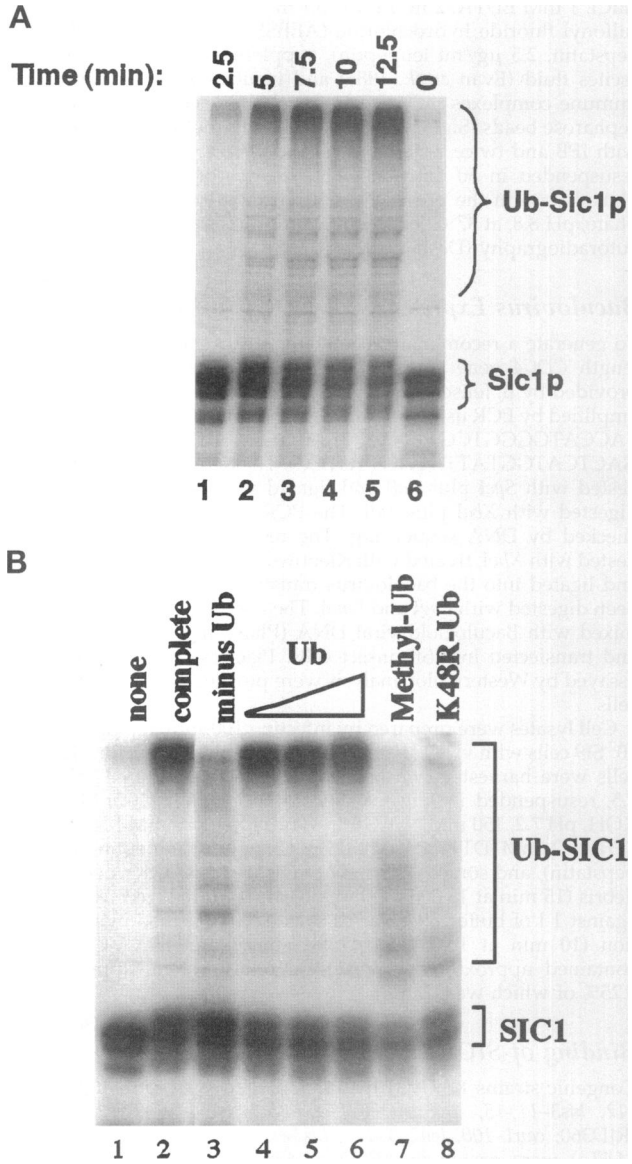

Figure 1. SIC1 is multiubiquitinated in vitro. (A) Time-dependent conversion of SIC1 to higher molecular mass forms by yeast extract. Extract from cells arrested in G_1 due to CLN depletion was fractionated by chromatography on DEAE resin and supplemented with ubiquitin (Ub), CDC34, GST-CLN2, and ARS. ^{35}S-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 multiubiquitination. 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 G_1-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 G_1 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. ^{35}S-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 arose from multiubiquitination: 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 multiubiquitin chain formation by preventing Ub-Ub ligation (Hershko and Heller, 1985; Chau *et al.*, 1989). In sum, the data in Figure 1 demonstrate that multiubiquitination of SIC1 in fractionated yeast extract was rapid, efficient, and highly processive. Indeed, the CDC34-dependent accumulation of multiubiquitinated 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 ubiquiti-

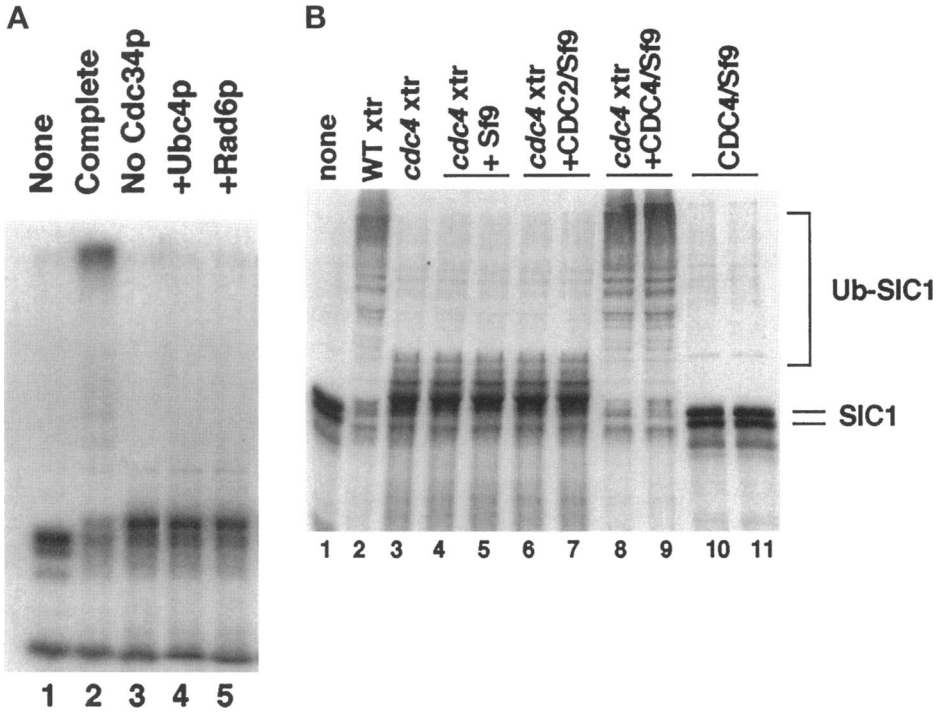

Figure 2. CDC34 and CDC4 are required for SIC1 multiubiquitination. (A) Ubiquitination of SIC1 requires CDC34. Enriched ^{35}S-labeled SIC1 translation product was incubated 15 min at 25°C in the absence (lane 1) or presence (lanes 2–5) of 0.25 M DEAE fraction supplemented with ubiquitin (Ub), ARS, and 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 μ g of RAD6 in the reactions shown in lanes 4 and 5, respectively. (B) Ubiquitination of SIC1 requires CDC4. ^{35}S-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 *cdc4*^{ts} (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.

nated species was produced upon incubation of SIC1 in unfractionated cell extract (our unpublished results). For the remainder of the experiments, we used fractionated cell extracts, however, as they were both more active for SIC1 ubiquitination and more versatile due to their dependence on CDC34 (see below).

Ubiquitination of SIC1 Requires CDC34 and CDC4

The disappearance of SIC1 at the G_1–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 [^{35}S]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 G_1-arrested *cdc4*^{ts} mutant cells. In contrast to the results obtained with a 0.25 M DEAE fraction prepared from wild-type cells (Figure 2B, lane

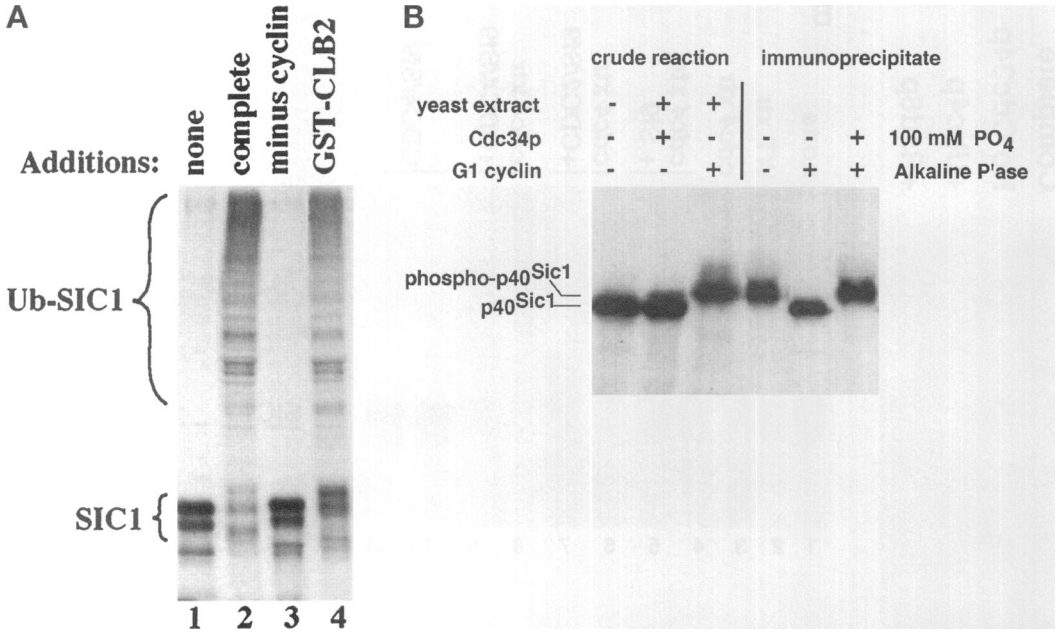

Figure 3. (A) Phosphorylation and ubiquitination of SIC1 require CLN2. In vitro ubiquitination reactions assembled in the absence (lane 1) or presence of the 0.25 M DEAE fraction prepared from *CLN*-depleted cells either were fully supplemented (lane 2) or were deprived of GST-CLN2 (lane 3). Lane 4 depicts a reaction in which GST-CLN2 was substituted for by 100 ng of GST-CLB2. (B) The modified form of SIC1 that accumulates in CDC34-deficient reactions is sensitive to phosphatase treatment. In vitro ubiquitination reactions containing ^{35}S-labeled SIC1$^{myc\text{-}His6}$ were conducted in the absence of CDC34, resulting in accumulation of SIC1$^{myc\text{-}His6}$ in an intermediate form (lane 3) as compared with reactions containing only translation product (lane 1) or reactions lacking GST-CLN2 (lane 2). An aliquot of modified SIC1$^{myc\text{-}His6}$ was immunoprecipitated (lanes 4 through 6) and treated with calf intestinal alkaline phosphatase (see MATERIALS AND METHODS) in the absence (lane 5) or presence (lane 6) of phosphate competitor, and samples were evaluated by SDS-PAGE and autoradiography.

2), the 0.25 M DEAE fraction isolated from *cdc4^{ts}* 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 *cdc4^{ts}* 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 *cdc34^{ts}* 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 multiubiquitination of SIC1, we assembled ubiquitination reactions lacking exogenously added GST-CLN2. Since the 0.25 M DEAE fraction was prepared from G_1-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 CDC28-associated protein kinase activity, as was reported for unfractionated extracts (Deshaies and Kirschner, 1995; our 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 G_1-arrested *cdc28^{ts}* 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 (SIC1^{mycHis6}) 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 immunoisolated 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 *cdc34^{ts}* cells as compared with *cdc28^{ts}* cells

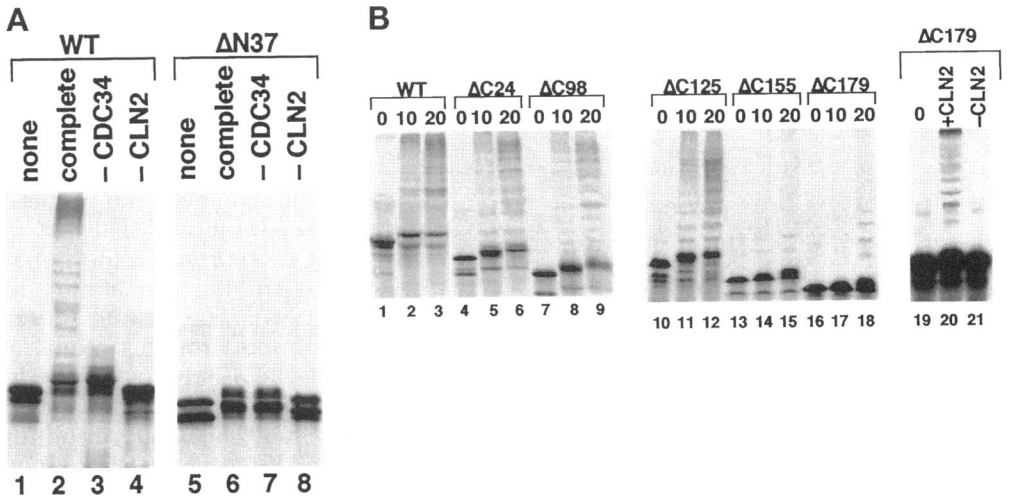

Figure 4. The N-terminal domain of SIC1 is both necessary and sufficient for ubiquitination. ^{35}S-labeled SIC1 truncation mutants generated by transcription/translation of mutant PCR-derived templates were incubated under standard reaction conditions with 0.25 M DEAE fraction supplemented with ARS, 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 ΔC refers to the number of codons deleted from the 3′ end (e.g., ΔC24 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 [^{35}S]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-

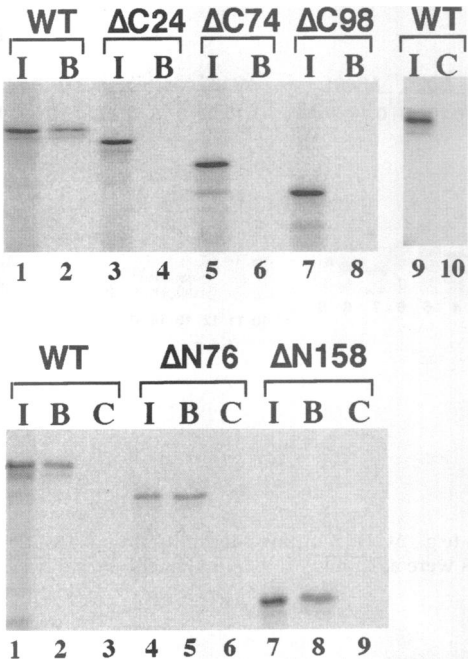

Figure 5. The C-terminal domain of SIC1 is both necessary and sufficient for binding to CLB5. ^{35}S-labeled SIC1 truncation mutants generated by transcription/translation of mutant PCR-derived templates were incubated with extract from yeast cells expressing either untagged or HA-tagged CLB5. CLB5HA-associated proteins were retrieved by immunoprecipitation with anti-HA monoclonal antibody 12CA5 and detected by SDS-PAGE followed by autoradiography. I refers to the input fraction, B refers to the bound fraction from reactions containing CLB5HA extract, and C refers to the bound fraction from reactions containing untagged CLB5 extract. In each set of lanes, the antibody-enriched fractions B and C were overloaded twofold with respect to the input fraction. Top, C-terminal truncation mutants deleted for the indicated number of amino acids from the C terminus (e.g., ΔC24 lacks amino acids 261–284). Bottom, N-terminal truncation mutants deleted for the indicated number of amino acids from the N terminus (e.g., ΔN 76 lacks amino acids 1–76).

nus of SIC1 (ΔC24) inactivated its ability to bind CLB5HA (Figure 5 top, lanes 3 and 4) but had little or no effect on SIC1 ubiquitination (Figure 4B, lane 6).

DISCUSSION

We report herein the reconstitution of SIC1 ubiquitination in extracts from budding yeast cells. Assembly of multiubiquitin chains on SIC1 in vitro required the E2 enzyme CDC34, a potential E3 subunit known as CDC4, and cyclin-dependent protein kinase activity. The N-terminal domain of SIC1 was both necessary and sufficient to direct CDC34- and cyclin-dependent ubiquitination of SIC1. In contrast, the C-terminal domain mediated the association of SIC1 with CLB5/CDC28.

Factors Required for SIC1 Ubiquitination

Previously, it had been shown in vivo that *cdc4^{ts}*, *cdc34^{ts}*, *cdc53^{ts}*, *skp1^{ts}*, and *CLN*-deficient mutants fail to initiate both S-phase and SIC1 proteolysis (Goebl *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^{ts}* mutant extracts were defective for SIC1 ubiquitination, we were unable to rescue them with Sf9 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.

Multiubiquitination 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) G_1 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 *cdc28^{ts}* 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 G_1–S Transition Work?

The observations reported herein raise several questions about the mechanism of the G_1–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 *sic1*Δ cells enter S-phase precociously (Schneider *et al.*, 1996), it seems plausible that there exists a window of time in G_1 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 *cdc34^{ts}* mutant block requires the continuous synthesis of CLN protein (Schneider *et al.*, 1996).

One factor that may contribute to the sharpness of the G_1–S transition in vivo is the potential for positive feedback in the degradation of SIC1. Since multiubiquitination 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 G_1–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

acids of SIC1 are absolutely essential for this interaction. It is interesting to note that this extreme C-terminal portion of SIC1 contains a copy of the "ZRXL" motif (KRRL), which has been implicated as a cyclin/CDK2 recognition determinant (Adams *et al.*, 1996). Mutant A- and B2-type cyclins that fail to bind CDC2 are poor substrates for ubiquitin-mediated proteolysis during anaphase (Stewart *et al.*, 1994). In contrast, a mutation (SIC1 ΔC24) that disrupts the interaction between SIC1 and CLB5/CDC28 has no discernible effect on SIC1 ubiquitination. Unlike cyclins, which are normally present in amounts substoichiometric with respect to CDC2, the concentration of SIC1 most likely exceeds that of CLB/CDC28 complexes in G_1 cells. That SIC1 can be ubiquitinated regardless of whether it is bound to CLB5/CDC28 would ensure that the entire pool of SIC1 is synchronously destroyed at the G_1–S transition, thereby preventing a pool of monomeric stable SIC1 from quenching the activity of CLB5/CDC28 complexes that had just been liberated by the destruction of bound SIC1 molecules.

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