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A Complex of Cdc4p, Skp1p, and Cdc53p/Cullin Catalyzes Ubiquitination of the Phosphorylated CDK Inhibitor Sic1p

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Summary

In S. cerevisiae, the G1/S transition requires Cdc4p, Cdc34p, Cdc53p, Skp1p, and the Cln/Cdc28p cyclin-dependent kinase (Cdk). These proteins are thought to promote the proteolytic inactivation of the S-phase Cdk inhibitor Sic1p. We show here that Cdc4p, Cdc53p, and Skp1p assemble into a ubiquitin ligase complex named SCF^{Cdc4p}. When mixed together, SCF^{Cdc4p} subunits, E1 enzyme, the E2 enzyme Cdc34p, and ubiquitin are sufficient to reconstitute ubiquitination of Cdk-phosphorylated Sic1p. Phosphorylated Sic1p substrate is specifically targeted for ubiquitination by binding to a Cdc4p/Skp1p subcomplex. Taken together, these data illuminate the molecular basis for the G1/S transition in budding yeast and suggest a general mechanism for phosphorylation-targeted ubiquitination in eukaryotes.

Introduction

A number of cellular processes in both prokaryotes and eukaryotes are controlled by selective proteolysis. Proteolysis in the eukaryotic cytosol typically involves the assembly of a substrate-linked ubiquitin chain, which targets the protein for degradation by the 26S proteasome (Hochstrasser, 1995). Ubiquitin is first activated at its C terminus through the formation of a thioester bond with the ubiquitin-activating enzyme, E1. Ubiquitin is subsequently transesterified to a member of a family of ubiquitin conjugating (E2) enzymes. Finally, ubiquitin is transferred from the E2 enzyme to a lysine residue of the target protein, either directly or with the assistance of a ubiquitin ligase (E3). E3 was originally defined as an activity that is both necessary and sufficient for the transfer of ubiquitin to a model substrate in the presence of a ubiquitin-charged E2 enzyme (Hershko et al., 1983). E3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as some E3s have been shown to bind directly to substrate. Recent work suggests that the E6-AP E3 (Huibregtse et al., 1993) also forms a thioester with ubiquitin as an intermediate in the transfer of ubiquitin from E2 to substrate (Scheffner et al., 1995). Since E3s do not possess detectable sequence homology, and since few have been characterized mechanistically, it remains to be seen whether all physiological ubiquitination reactions require E3s, and whether all E3s will participate directly in both substrate recruitment and ubiquitin transfer.

Entry into S phase in budding yeast requires the activity of S phase–promoting Clb/Cdc28p protein kinase complexes (Schwob et al., 1994). Although these complexes are assembled during G1 phase, they are initially inactive due to the presence of high levels of the Clb/Cdc28p inhibitor Sic1p (Nugroho and Mendenhall, 1994; Schwob et al., 1994). At the G1/S transition, Sic1p is abruptly degraded and the liberated Clb/Cdc28p complexes trigger DNA synthesis. *CLN1–3*, *CDC4*, *CDC34*, *CDC53*, and *SKP1* are required for the timely disappearance of Sic1p in vivo (Schwob et al., 1994; Bai et al., 1996; Schneider et al., 1996). Since *CDC34* encodes an E2 enzyme, it was proposed that Sic1p degradation is mediated by ubiquitin-dependent proteolysis (Schwob et al., 1994). Indeed, Sic1p is efficiently ubiquitinated in yeast extract, and Cdc34p, Cdc4p, and Cln/Cdc28p are required for this process (Verma et al., 1997b). Biochemical and genetic experiments have revealed that the main role of Cln/Cdc28p in the G1/S transition is to phosphorylate Sic1p, which triggers its ubiquitination by the Cdc34p pathway (Verma et al., 1997c; Schneider et al., 1996; Tyers, 1996).

Although Cdc4p and Cdc34p have been implicated in Sic1p ubiquitination in yeast extract, it is not known whether Skp1p and Cdc53p are also directly involved in this process. The functional roles of these proteins are of great interest, since Skp1p and Cdc53p are members of multigene families that are conserved from yeast to humans (Kipreos et al., 1995; Zhang et al., 1995; Bai et al., 1996; Mathias et al., 1996). Skp1p and Cdc53p homologs have been implicated in diverse processes, including kinetochore assembly in yeast (Connelly and Hieter, 1996), withdrawal from the cell cycle in nematodes (Kipreos et al., 1995), and both S phase progression (Zhang et al., 1995) and transcription elongation (Pause et al., 1997) in animal cells. Here, we report the reconstitution of Sic1p ubiquitination with a set of purified proteins, including Cdc4p, Cdc53p, and Skp1p. We also show that Cdc4p, Cdc53p, and Skp1p assemble into an E3 complex that selectively recognizes phosphorylated Sic1p via its Cdc4p and Skp1p subunits. These findings suggest that phosphorylation-triggered ubiquitin-dependent proteolysis may be mediated by a conserved cullin-dependent pathway in eukaryotic cells.

Results

Reconstitution of Sic1p Ubiquitination in Insect Cell Lysates

In vitro reconstitution of Sic1p ubiquitination using fractionated yeast extract revealed a direct requirement for Cdc34p, Cdc4p, and cyclin-dependent kinase (Cdk) activities (Verma et al., 1997b). However, yeast extract contributed a host of unidentified components, and a defined set of proteins sufficient to sustain Sic1p ubiquitination remained elusive. To define the biochemical

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requirements for Sic1p ubiquitination, several proteins that had been implicated in Sic1p destruction—including Cln2p/Cdc28p complexes (Reynard and Deshaies, unpublished data), Cdc53p, Cdc4p (Verma et al., 1997b), and Skp1p—were expressed separately in Sf9 insect cells via infection with recombinant baculoviruses. Previously described recombinant expression systems were used to prepare the yeast E1 enzyme ^{His6}Uba1p (Dohmen et al., 1995; Figure 2A, lane 1), the E2 enzyme Cdc34p (Banerjee et al., 1993; see Figure 2A lane 2), and a maltose-binding protein-Sic1p chimera containing a C-terminal bipartite Myc epitope–hexahistidine tag (MBP-Sic1p^{MH6}) (Verma et al., 1997a). Purified, metabolically radiolabeled [^{35}S]-MBP-Sic1p^{MH6} is ubiquitinated in fractionated yeast extract similarly to wild-type Sic1p produced by in vitro translation (Verma et al., 1997b). We used monomeric [^{35}S]-MBP-Sic1p^{MH6} as a substrate for most of the experiments reported here because previous work indicated that association of Sic1p with Clb5p/Cdc28p was not required for its ubiquitination (Verma et al., 1997b).

To test whether this set of recombinant proteins was sufficient to direct ubiquitination of Sic1p in the absence of other yeast proteins, we mixed unfractionated insect cell lysates containing Cdc4p, Cdc53p, and Skp1p with purified ^{His6}Uba1p, Cdc34p, ubiquitin, and an ATP-regenerating system. For the direct phosphorylation of Sic1p, which is essential for its ubiquitination (Verma et al., 1997c), we also added affinity-purified Cln2p/Cdc28p^{HA}/Cks1p protein kinase complexes to the reaction. In work to be published elsewhere (Reynard and Deshaies, unpublished data), we demonstrated that Cln2p/Cdc28p protein kinase activity is dependent upon the budding yeast p13^{suc1} homolog Cks1p (Hadwiger et al., 1989). When mixed with the components listed above, [^{35}S]-MBP-Sic1p^{MH6} was converted to a ladder of high molecular weight (HMW) species (Figure 1A, lane 8). The HMW MBP-Sic1p^{MH6} species generated in this reaction represent the assembly of substrate-linked multiubiquitin chains, since their formation was markedly reduced by the omission of ubiquitin (lane 1; see also Figure 3). Reactions lacking Cdc4p, Cdc34p, Cdc53p, or Skp1p (lanes 3–6) failed to generate HMW ubiquitin conjugates and instead yielded phosphorylated Sic1p. In contrast, Sic1p was neither phosphorylated nor ubiquitinated in reactions lacking Cln2p/Cdc28p/Cks1p (lane 2). The requirement for Cdk activity could be satisified by adding either Cln2p/Cdc28p/Cks1p or Clb5p/Cdc28p complexes (Figure 1B, lane 1 versus lane 4). This suggests that newly liberated Clb5p/Cdc28p complexes may contribute to the destruction of Sic1p in vivo, thereby sharpening the G1/S transition (Verma et al., 1997b). Surprisingly, HMW derivatives of MBP-Sic1p^{MH6} were efficiently generated in the absence of ^{His6}Uba1p (lane 7). This presumably reflects the fact that a conserved Uba1p-like activity is most likely present in insect cell lysates, since reactions utilizing purified components demonstrated a direct requirement for ^{His6}Uba1p (see below, Figure 3). Note that the C-terminal 60 amino acids of Cdc53p, which are highly conserved among eukaryotic cullins (Kipreos et al., 1995; Mathias et al., 1996), were not required for the ubiquitination-promoting activity of Cdc53p (lane 9).

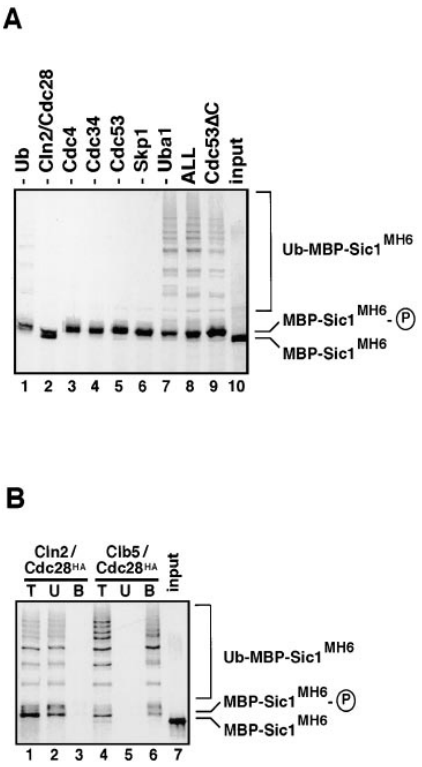

Figure 1. Ubiquitination of MBP-Sic1p In Vitro Requires Cdc4p, Cdc34p, Cdc53p, Skp1p, and Cyclin-Dependent Kinase

(A) Unfractionated lysates from baculovirus-infected Sf9 cells expressing Cdc4p, Cdc53p, and Skp1p, and purified Cdc34p, ^{His6}Uba1p, and Cln2p/Cdc28p^{HA}/Cks1p complexes were prepared as described in Experimental Procedures. The substrate MBP-Sic1p^{MH6} was expressed and metabolically labeled in E. coli and purified via affinity chromatography (lane 10). Substrate was mixed with all of the above components plus ubiquitin and an ATP-regenerating system (lane 8). For lanes 1–7, the component indicated above each lane was omitted from the reaction mixture. In lane 9, Cdc53p lysate was replaced by an insect cell lysate containing Cdc53pΔC, a mutant of Cdc53p lacking its 60 C-terminal residues. All reactions were incubated at 25°C for 90 minutes, stopped by addition of SDS–PAGE buffer, resolved by SDS–PAGE (8%), and visualized by autoradiography. The positions of unmodified, phosphorylated, and ubiquitinated MBP-Sic1p^{MH6} are indicated.

(B) In vitro ubiquitination reactions containing either immunopurified Cln2p/Cdc28p^{HA}/Cks1p (lane 1) or Clb5p/Cdc28p^{HA} (lane 4) as a source of kinase were assembled as described in Figure 1. Lane 7 contains input MBP-Sic1p^{MH6} used in the ubiquitination reactions. After incubation at 25°C for 1 hour, Cdc28p^{HA} and associated proteins were immunoprecipitated with the anti-HA antibody 12CA5. Equivalent amounts of the total reactions ([T], lanes 1 and 4) unbound ([U], lanes 2 and 5) and 12CA5-bound ([B], lanes 3 and 6) fractions were resolved by SDS–PAGE, and MBP-Sic1p^{MH6} was visualized by autoradiography.

Although the ubiquitination of Sic1p leads to its destruction and the subsequent release of Clb5p/Cdc28p from inhibition, it is unclear if the ubiquitination of Sic1p is sufficient to dislodge it from Clb5p/Cdc28p. To address this question, the reactions shown in Figure 1B, lanes 1 and 4 were incubated with anti-HA antibody to immunoprecipitate Cdc28p^{HA} and associated proteins. As expected, MBP-Sic1p^{MH6} did not bind Cln2p/Cdc28p^{HA}/

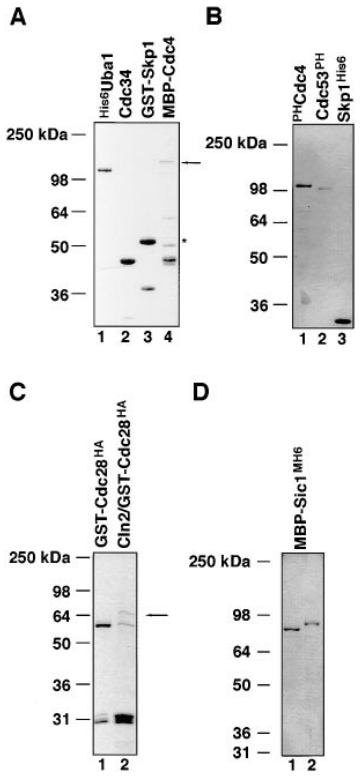

Figure 2. Purified Ubiquitination Components

(A–D) Recombinant proteins purified from yeast (^{His6}Uba1p), E. coli (GST-Skp1p, MBP-Cdc4p, MBP-Sic1p^{MH6}), and baculovirus-infected Sf9 cells (^{PH}Cdc4p, Cdc53p^{PH}, Cln2p/GST-Cdc28p^{HA}, GST-Cdc28p^{HA}, Skp1p^{His6}) as described in Experimental Procedures were separated on SDS–polyacrylamide gels and stained with Coomassie blue. In (A), the asterisks and arrowhead denote full-length GST-Skp1p (lane 3) and MBP-Cdc4p (lane 4), respectively. In (C), Cln2p is designated by an arrow. In (D), purified MBP-Sic1p^{MH6} was incubated with bead-bound Cln2p/Cdc28p^{HA}/Cks1p kinase in the absence (lane 1) or presence (lane 2) of ATP. These forms of substrate were used for the experiments shown in Figure 4.

Cks1p (lane 3); in contrast, both unmodified and multiubiquitinated MBP-Sic1p^{MH6} were both efficiently coimmunoprecipitated with Clb5p/Cdc28p^{HA} (lane 6).

Reconstitution of MBP-Sic1p^{MH6} Ubiquitination with Purified Proteins

The experiment shown in Figure 1A clearly established that Cdc4p, Cdc53p, Skp1p, and Cdc34p were required for the ubiquitination of MBP-Sic1p^{MH6}. However, these experiments were conducted with unfractionated insect cell lysates that contained E1 activity, ubiquitin, and potentially other, unidentified ubiquitination factors. To define a set of components that are both necessary and sufficient for the ubiquitination of Sic1p, we individually expressed and purified the recombinant Cdc4p, Cdc53p, and Skp1p proteins. Glutathione-S-transferase-Skp1p (GST-Skp1p) was expressed in E. coli and purified by glutathione affinity chromatography (Figure 2A, lane 3). ^{PH}Cdc4p (Cdc4p tagged at its N terminus with a bipartite Polyoma antigen–Hemagglutinin epitope, hereafter denoted as PH), Cdc53p^{PH} (Cdc53p tagged with a PH epitope at its C terminus), and Skp1p^{His6} were expressed in Sf9 insect cells via infection with recombinant baculoviruses and affinity-purified on either anti-polyoma monoclonal antibody or Ni^{+}-NTA matrices (Figure 2B, lanes 1–3). Cln2p/GST-Cdc28p^{HA} complexes were produced in Sf9 insect cells via coinfection with recombinant baculoviruses, activated by addition of purified Cks1p, and affinity-purified on glutathione resin (Figure 2C, lane 2). Purification of GST-Cdc28p^{HA} alone from Sf9 cells is shown for comparison (lane 1). Similar results were obtained with Cln2p/Cdc28p^{HA}/Cks1p complexes purified on an anti-HA monoclonal antibody matrix (data not shown).

The availability of purified proteins allowed us to test whether this set of components was sufficient to direct ubiquitination of MBP-Sic1p^{MH6}. Since the principal function of Cdk complexes in this reaction is to phosphorylate Sic1p (Verma et al., 1997c), we prepared competent substrate by mixing purified [^{35}S]-MBP-Sic1p^{MH6} with ATP and Cln2p/GST-Cdc28p^{HA}/Cks1p complexes that were bound to glutathione-agarose beads. Following a brief incubation, the soluble pool of phosphorylated substrate was separated from the beads. When phosphorylated [^{35}S]-MBP-Sic1p^{MH6} was mixed with purified ^{PH}Cdc4p, Cdc34p, Cdc53p^{PH}, GST-Skp1p, ^{His6}Uba1p, ubiquitin, and an ATP-regenerating system, it was extensively ubiquitinated (Figure 3A, lane 8). Substituting ubiquitin with the chain-terminating derivatives methyl-ubiquitin (Hershko and Heller, 1985) or K48R-ubiquitin (Chau et al., 1989; Figure 3B), or omitting ubiquitin from the reaction entirely (Figure 3A, lane 6) dramatically altered the profile of MBP-Sic1p^{MH6} modification. These results confirm that the HMW forms observed in Figure 3A, lane 8 correspond to multiubiquitinated MBP-Sic1p^{MH6}.

As was observed with crude components (Figure 1A), no ubiquitination was detected when ^{PH}Cdc4p, Cdc34p, or Cdc53p^{PH} were omitted from the reaction (Figure 3A, lanes 2–4), or when unphosphorylated MBP-Sic1p^{MH6} was used as substrate (lane 1). In contrast to reactions containing crude lysate, reactions carried out with purified components were absolutely dependent upon ^{His6}Uba1p (lane 7).

Unexpectedly, omitting GST-Skp1p from the reaction mixture did not affect the ubiquitination of MBP-Sic1p^{MH6} (Figure 3A, lane 5). This was in marked contrast to the clear requirement for Skp1p seen in the previous experiments using crude insect cell lysates (Figure 1A). Previous studies have identified a specific interaction between Cdc4p and Skp1p that is mediated by a domain in Cdc4p known as the F box (Bai et al., 1996). Skp1p appears to be conserved in eukaryotes, as homologs have been identified in A. thaliana, C. elegans, and humans (Zhang et al., 1995; Bai et al., 1996). We reasoned that if an insect Skp1p-like protein was present, it might efficiently bind to and copurify with ^{PH}Cdc4p, and be present in sufficient quantity to provide a detectable activity in our ubiquitination assay.

To produce Cdc4p devoid of contaminating Skp1p activity, an MBP-Cdc4p fusion was expressed in E. coli and purified by amylose affinity chromatography (Figure 2A, lane 4). Purified MBP-Cdc4p was able to substitute for ^{PH}Cdc4p in a ubiquitination reaction conducted with purified components (Figure 3C, lane 3). However, in

contrast to the results obtained with ^{PH}Cdc4p (Figure 3A, lane 5), reactions conducted with MBP-Cdc4p were absolutely dependent upon GST-Skp1p (Figure 3C, lane 2). Since Skp1p^{His6} remains active in promoting ubiquitination even after being heated at 95°C (data not shown), we hypothesized that any insect Skp1p that copurified with ^{PH}Cdc4p would dissociate from heat-denatured ^{PH}Cdc4p but would retain its activity. Whereas a heat-treated preparation of ^{PH}Cdc4p no longer possessed Cdc4p activity (data not shown), it partially fulfilled the requirement for Skp1p activity (lane 4). Thus, ^{PH}Cdc4p copurifies from insect cells with a heat-stable Skp1p-like activity that can promote the ubiquitination of Sic1p. It remains unclear why this Skp1p-like activity failed to sustain MBP-Sic1p^{MH6} ubiquitination in crude Sf9 cell lysates in the absence of exogenously added Skp1p. Nevertheless, our data suggest that Cdc34p, Cdc4p, Cdc53p, and Skp1p, along with ^{His6}Uba1p, ubiquitin, and ATP, are necessary and sufficient to support efficient multiubiquitination of phosphorylated Sic1p. Since Cdc4p, Cdc53p, and Skp1p are required to direct the Cdc34p-dependent ubiquitination of phospho-Sic1p, these proteins appear to function collectively as an E3.

Phosphorylated MBP-Sic1p^{MH6} Is Specifically Targeted for Ubiquitination by Interaction with Cdc4p/Skp1p

In the two ubiquitination pathways that have been best characterized to date—the N-end rule and the E6/E6-AP pathway—substrate selectivity is conferred by a specific interaction between the substrate and an E3 (Bartel et al., 1990; Scheffner et al., 1993). Since Cdc4p, Cdc53p, and Skp1p collectively serve as an E3, we hypothesized that one of these proteins might interact selectively with phosphorylated Sic1p. To test this possibility, we performed coimmunoprecipitation experiments using either unphosphorylated or phosphorylated MBP-Sic1p^{MH6} (Figure 2D) as bait. Sf9 cells singly infected with baculoviruses expressing either Cdc4p, Cdc53p, or Skp1p were metabolically labeled with ^{35}S-amino acids, and lysates derived from the labeled cells (Figure 4A, lanes 1 and 4; Figure 4B, lane 1) were incubated separately with either phosphorylated or unmodified MBP-Sic1p^{MH6}, after which MBP-Sic1p^{MH6} and associated proteins were recovered with anti-MBP antibodies. This analysis revealed that ^{PH}Cdc4p (Figure 4A, lanes 2 and 3), but not Cdc53p (lanes 5 and 6) or Skp1p^{HA} (Figure 4B, lanes 2 and 3), selectively bound to phosphorylated MBP-Sic1p^{MH6}. Combining Cdc53p- and Skp1p^{HA}-containing lysates did not result in enhanced interaction of these components with either form of MBP-Sic1p^{MH6} (data not shown). In contrast, when Skp1p^{HA} lysate was mixed with ^{PH}Cdc4p lysate, both proteins were selectively coimmunoprecipitated with phosphorylated MBP-Sic1p^{MH6}, and the level of coprecipitated ^{PH}Cdc4p was substantially increased (compare Figure 4B, lane 5 with Figure 4A, lane 3).

Mutational analysis of Cdk phosphoacceptor sites in Sic1p revealed that phosphorylation of a specific set of residues triggers Sic1p destruction. An epitope-tagged version of Sic1p lacking these key Cdk phosphoacceptor sites (Sic1p^{HAHis6}-T5G, T33A, S76A; Sic1p^{HAHis6}-Δ3P)

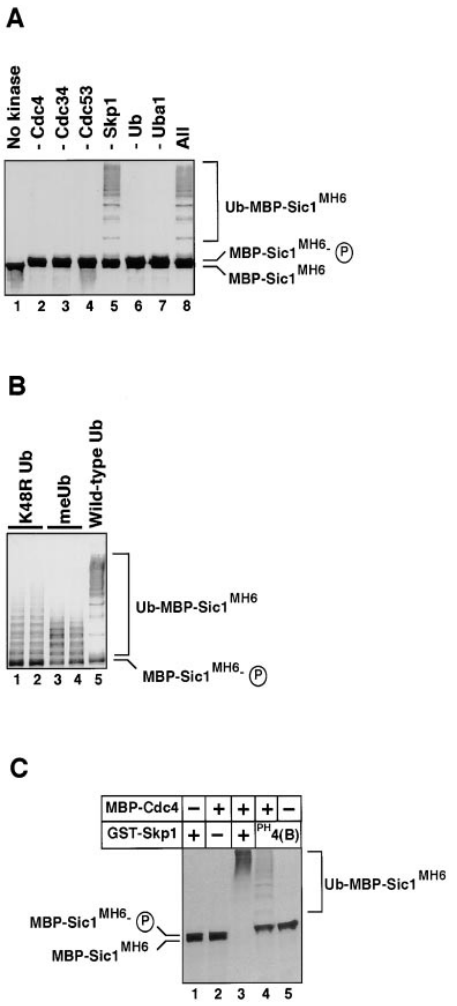

Figure 3. Reconstitution of MBP-Sic1p^{MH6} Ubiquitination with Purified Proteins.

(A) Purified ^{PH}Cdc4p, Cdc34p, Cdc53p^{PH}, GST-Skp1p, ^{His6}Uba1p, ubiquitin, [^{35}S]-MBP-Sic1p^{MH6} that had been phosphorylated by incubation with immobilized Cln2p/GST-Cdc28p^{HA}/Cks1p complexes, and an ATP-regenerating system were mixed together (lane 8). Reactions shown in lanes 2–7 lack the indicated component. For lane 1, all components listed above were added to MBP-Sic1p^{MH6} that had not been preincubated with Cln2p/GST-Cdc28p^{HA}/Cks1p. Reactions were incubated at 25°C for 90 minutes, stopped by addition of SDS–PAGE buffer, resolved by SDS–PAGE (8%), and visualized by autoradiography. The positions of MBP-Sic1p^{MH6}, phosphorylated MBP-Sic1p^{MH6}, and ubiquitinated MBP-Sic1p^{MH6} are indicated.

(B) Reactions were assembled using purified proteins as described in part (A), except that reactions contained either mutant K48R ubiquitin (lanes 1 and 2), methyl ubiquitin (lanes 3 and 4), or wild-type ubiquitin (lane 5). Reactions were incubated and processed as described above.

(C) In all reactions, [^{35}S]-MBP-Sic1p^{MH6} was incubated with soluble Cln2p/GST-Cdc28p^{HA}/Cks1p, ^{His6}Uba1p, Cdc34p, and Cdc53p^{PH} along with ubiquitin and an ATP-regenerating system as described above. In addition, the following components were added to the indicated reactions: GST-Skp1p (lane 1), MBP-Cdc4p (lane 2), MBP-Cdc4p plus GST-Skp1p (lane 3), and the soluble fraction recovered from heat-denatured preparations of purified ^{PH}Cdc4p either with (lane 4) or without MBP-Cdc4p (lane 5). All reactions were incubated at 24°C for 45 min and then evaluated by SDS–PAGE and autoradiography.

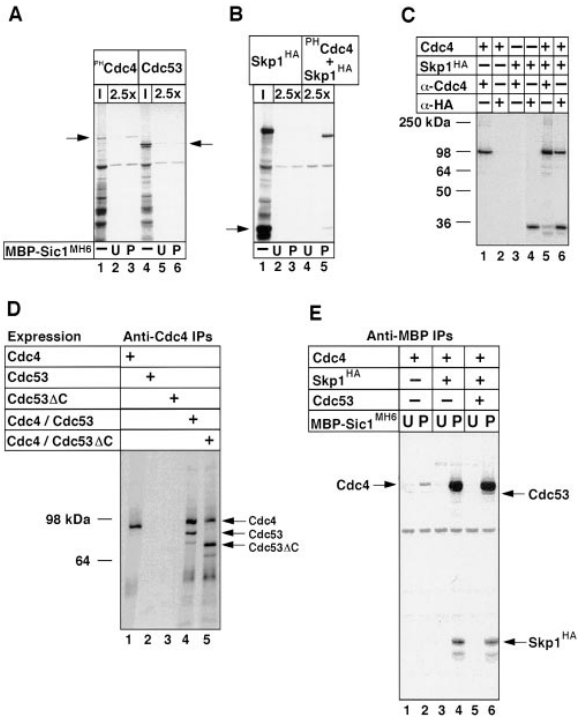

Figure 4. Phosphorylated MBP-Sic1p^{MH6} Specifically Binds to Cdc4p/Skp1p and Assembles into a Complex with Cdc4p, Skp1p, and Cdc53p

Sf9 cells were infected with the indicated baculovirus and metabolically labeled with Tran[^{35}S]-label for 3 hr. For (A), (B), and (E), the derived radiolabeled lysates were then incubated with either unphosphorylated (U) or phosphorylated (P) MBP-Sic1p^{MH6}, and following a 1 hr incubation at 4°C MBP-Sic1p^{MH6}-associated proteins were immunoprecipitated with anti-MBP antibodies and analyzed by SDS–PAGE, followed by autoradiography. The input (I) lanes were underloaded 2.5-fold relative to the immunoprecipitates. (A) and (B) were from a single exposure of the same gel and so can be compared directly with each other.

(A) Radiolabeled lysates containing ^{PH}Cdc4p (lanes 2 and 3) or Cdc53p (lanes 5 and 6) were used for analysis. The relative position of each protein is indicated by an arrow.

(B) Radiolabeled lysate containing Skp1p^{HA} (lanes 1–3) or an equal mixture of Skp1p^{HA} plus ^{PH}Cdc4p-containing lysates (lanes 4 and 5) was used.

(C) Radiolabeled lysates containing Cdc4p, Skp1p^{HA}, or a mixture of the two were immunoprecipitated with either anti-Cdc4p or anti-HA antibodies as indicated.

(D) Sf9 cells were singly infected with baculoviruses expressing Cdc4p (lane 1), Cdc53p (lane 2), and Cdc53pΔC (lane 3), or coinfected with Cdc4p and Cdc53p (lane 4) or Cdc4p and Cdc53pΔC (lane 5) baculoviruses. [^{35}S]-radiolabeled lysates were prepared from each infection and immunoprecipitated with anti-Cdc4p antibodies. The immunoprecipitates were analyzed by SDS–PAGE followed by autoradiography.

(E) Radiolabeled lysates containing Cdc4p (lanes 1–6) were mixed with radiolabeled Skp1p^{HA} (lanes 3–6) or Cdc53p (lanes 5 and 6) lysates prior to addition of MBP-Sic1p^{MH6}, followed by immunoprecipitation using anti-MBP antibodies.

was neither ubiquitinated in vitro nor degraded in vivo, but retained the ability of the wild-type protein to bind to Clb5p/Cdc28p complexes (Verma et al., 1997c). Given the results described above, we surmised that the stability of the Sic1p^{HAHis6}-Δ3P mutant might arise from a failure to bind to Cdc4p/Skp1p. To test this hypothesis, we

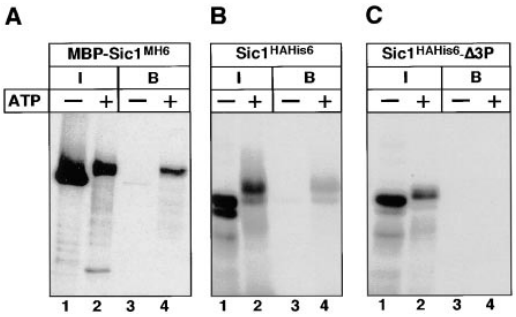

Figure 5. Purified Cdc4p Specifically Binds Phosphorylated Sic1p but Not a Stabilized Mutant Form of Sic1p

[^{35}S]-radiolabeled Sic1p proteins were incubated with purified bead-bound Cln2p/GST-Cdc28p^{HA}/Cks1p in the absence (−) or presence (+) of ATP to produce either an unphosphorylated or phosphorylated form of each protein. Following the kinase reactions, the supernatant fractions containing [^{35}S]-Sic1p were withdrawn and mixed with bead-bound purified ^{PH}Cdc4p. After a 1 hr incubation, the ^{PH}Cdc4p beads were recovered and analyzed by SDS–PAGE followed by autoradiography. The input lanes were underloaded 1.5-fold relative to the bead-bound fractions.

(A) [^{35}S]-MBP-Sic1p^{MH6} was purified from metabolically labeled E. coli.

(B) [^{35}S]-Sic1p was produced by in vitro translation in rabbit reticulocyte lysate.

(C) [^{35}S]-Sic1p-Δ3P was produced by in vitro translation in rabbit reticulocyte lysate. The Sic1p-Δ3P mutant lacks three Cln2p/Cdc28p phosphoacceptor sites and is neither ubiquitinated in vitro nor degraded in vivo (Verma et al., 1997c).

affinity-purified ^{PH}Cdc4p from insect cell lysates using an anti-polyoma antibody matrix and incubated the immobilized ^{PH}Cdc4p complexes with various [^{35}S]-labeled forms of Sic1p. Incubation of the bead-bound ^{PH}Cdc4p with either unphosphorylated or phosphorylated MBP-Sic1p^{MH6} confirmed that only the phosphorylated form bound to the ^{PH}Cdc4p matrix (Figure 5A). Sic1p^{HAHis6} produced by in vitro translation in rabbit reticulocyte lysate also bound to ^{PH}Cdc4p-containing beads in a phosphorylation-dependent manner (Figure 5B), but Sic1p^{HAHis6}-Δ3P was not retained by the ^{PH}Cdc4p matrix even though it was clearly phosphorylated by Cln2p/Cdc28p^{HA}/Cks1p on sites whose modification is not sufficient to promote ubiquitination (Verma et al., 1997c; Figure 5C). These data establish two key points: first, the stability of Sic1p^{HAHis6}-Δ3P can be explained by its inability to bind Cdc4p/Skp1p; second, the failure of Cdc4p/Skp1p to interact with stable (yet phosphorylated) Sic1p^{HAHis6}-Δ3P suggests that the interaction between Cdc4p/Skp1p and phosphorylated wild-type Sic1p is physiologically significant and is not a nonspecific ionic interaction stabilized by the phosphate groups.

Recruitment of Phosphorylated Sic1p to a Skp1p/Cdc53p/Cdc4p E3 Complex

Cdc4p and Skp1p associate with one another in reticulocyte lysate (Bai et al., 1996). Consistent with this observation, recombinant Cdc4p and Skp1p expressed in insect cells also associated with each other, as judged by coimmunoprecipitation of Skp1p with Cdc4p (Figure 4C, lane 5) and vice versa (lane 6). Since Cdc4p assembled into complexes with both Skp1p and phosphorylated MBP-Sic1p^{MH6} in insect cell lysates, we also exam-

ined whether it could associate with Cdc53p. Sf9 cells that were either singly infected or coinfected with baculoviruses expressing Cdc4p, Cdc53p, or Cdc53pΔC were metabolically labeled with ^{35}S-amino acids, and lysates derived from the labeled cells were immunoprecipitated with anti-Cdc4p antibodies. As shown in Figure 4D, both Cdc53p (lane 4) and Cdc53p-ΔC (lane 5) specifically coprecipitated with Cdc4p. Since Cdc4p appeared to associate with a Skp1p-like activity in insect cell lysates (Figure 3C), it remains unclear whether Cdc4p and Cdc53p directly bound each other or interacted indirectly via Skp1p.

Given that Cdc4p can associate with Skp1p, Cdc53p, and phosphorylated substrate, and that the interaction between phosphorylated MBP-Sic1p^{MH6} and Cdc4p was strongly stimulated by Skp1p, we hypothesized that Cdc4p/Skp1p might serve as a receptor module that tethers phosphorylated substrate to an E3 complex containing Cdc53p. To test this possibility, we combined ^{35}S-radiolabeled Sf9 cell lysates containing Cdc4p, Cdc53p, and Skp1p^{HA} and incubated these mixtures with either phosphorylated or unmodified MBP-Sic1p^{MH6}, followed by immunoprecipitation with anti-MBP antibodies (Figure 4E). Whereas Cdc4p, Cdc53p, and Skp1p^{HA} did not coimmunoprecipitate with unphosphorylated MBP-Sic1p^{MH6}, all three proteins stably associated with phosphorylated MBP-Sic1p^{MH6}. Since Cdc53p by itself failed to bind selectively to phosphorylated MBP-Sic1p^{MH6} (Figure 4A, lane 6), the observed interaction was most likely due to the ability of both Cdc53p and phosphorylated MBP-Sic1p^{MH6} to bind independently to Cdc4p/Skp1p to form a single complex. Cdc53p has been reported to bind to Cdc34p (Willems et al., 1996). Thus, phosphorylated MBP-Sic1p^{MH6} bound to the Cdc4p/Cdc53p/Skp1p complex would be well-positioned to serve as a substrate for Cdc34p-dependent ubiquitination.

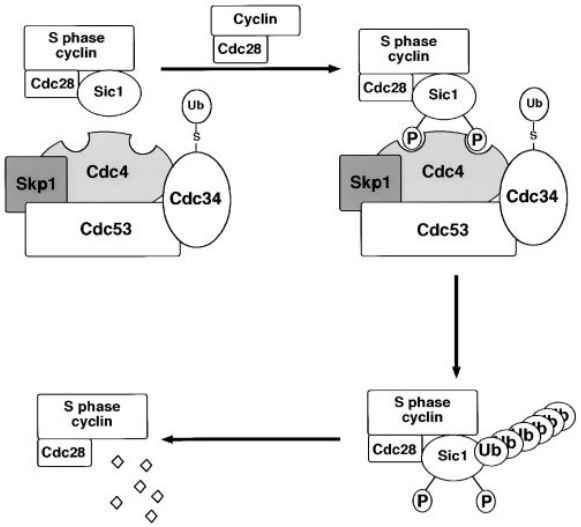

Figure 6. A Model for the Ubiquitination of Sic1p

In early G1 phase, Sic1p is bound to S-phase cyclin/Cdk complexes. For Sic1p to be destroyed, it must first be phosphorylated on several phosphoacceptor sites by an active cyclin/Cdk complex. Once phosphorylated, Sic1p is recognized by Cdc4p/Skp1p, which can interact with Cdc53p and thereby promote the transfer of a ubiquitin moiety from Cdc34p to substrate. Once ubiquitination has been initiated, multiubiquitin chains are processively elongated by a process that is not well understood, and the multiply ubiquitinated Sic1p is destroyed by the 26S proteasome. The exact nature of the protein–protein interactions between Cdc4p, Skp1p, Cdc53p, and Cdc34p implied by this figure has not yet been fully delineated.

Discussion

Reconstitution of Sic1p Ubiquitination with Purified Proteins

Ubiquitin-mediated degradation of the S phase Cdk inhibitor Sic1p plays a key role in triggering the initiation of DNA synthesis in budding yeast (Verma et al., 1997c; Schwob et al., 1994). Here, we report the ubiquitination of Sic1p using nine purified proteins: Uba1p, ubiquitin, Cdc34p, Cdc4p, Cdc53p, Skp1p, and Cln2p/Cdc28p/Cks1p complexes. Based on experiments reported here and elsewhere (Verma et al., 1997c; Willems et al., 1996), we envision the following sequence of events for Sic1p ubiquitination (Figure 6). Active Cln2p/Cdc28p/Cks1p complexes phosphorylate Sic1p on a set of Cdk consensus phosphorylation sites (Verma et al., 1997c), thereby priming it to serve as a substrate for ubiquitination. The phosphorylated Sic1p then binds to a complex containing Cdc4p, Cdc53p, and Skp1p subunits. We have agreed with Skowyra et al. (1997 [this issue of *Cell*]) to designate this assemblage SCF, which stands for Skp1p/Cdc53p (or Cullin)/F-box ubiquitin ligase complex, with the identity of the associated F-box protein indicated by a superscript (e.g., SCF^{Cdc4p}). Upon binding phosphorylated Sic1p, we propose that the Cdc53p subunit of SCF^{Cdc4p} recruits the ubiquitin-charged E2 enzyme Cdc34p to initiate Sic1p ubiquitination (Willems et al., 1996).

Several aspects of our model for Sic1p ubiquitination and degradation remain speculative. First, the interactions between Cdc4p, Cdc53p, Skp1p, Cdc34p, and phosphorylated Sic1p remains to be characterized in detail. Second, it is unclear how ubiquitin is transferred from Cdc34p to lysine residues in the SCF-bound substrate. Third, whereas SCF^{Cdc4p}, Cdc34p, E1 enzyme, and ubiquitin appear to be sufficient to sustain the ubiquitination of phosphorylated Sic1p in vitro, it is possible that additional proteins may regulate Sic1p ubiquitination in vivo. Such accessory factors might serve to activate known SCF^{Cdc4p} subunits or to overcome negative regulation that is not reconstituted in our in vitro system. Finally, it is unclear if SCF^{Cdc4p} rapidly dissociates from multiubiquitinated substrate or remains bound, perhaps interacting with the proteasome to facilitate the degradation of multiubiquitinated Sic1p.

Cdc4p/Skp1p Recruits Phosphorylated Substrates for Ubiquitination

A hallmark of SCF^{Cdc4p}-dependent ubiquitination is that it is selective for phosphorylated substrates. Cdc6p, Gcn4p, Far1p, and Sic1p are ubiquitinated in crude yeast extract in a Cdc34p- and Cdc4p-dependent manner, and in each case ubiquitination requires a kinase that phosphorylates the substrate (Verma et al., 1997c; Y. Chi and R. J. D., unpublished data). We show here that

this requirement derives from a selective interaction between phosphorylated substrate and Cdc4p/Skp1p. Skp1p by itself cannot bind to phospho-Sic1p. Whereas Cdc4p can interact with phospho-Sic1p in insect cell lysates, this interaction is enhanced considerably by Skp1p. The existence of a Skp1p-like activity in insect cells that copurifies with Cdc4p obscures whether phospho-Sic1p can be recognized by Cdc4p alone or only by a Cdc4p/Skp1p complex.

What are the individual roles of Cdc4p and Skp1p in substrate binding? Based on the observation that the WD-40 domains of β-transducin fold into a β-propeller structure with an extensive surface area suitable for protein–protein interaction (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996), we propose that the WD-40 domain of Cdc4p binds directly to phospho-Sic1p and that this interaction is facilitated by Skp1p. An unambiguous test of this hypothesis will likely require the generation of point mutations in the WD-40 repeats, since crude deletions in this domain appear to destabilize Cdc4p; Cdc4p-ΔWD mutants translated in reticulocyte lysate bind poorly to Skp1p and exhibit high nonspecific adsorption to matrices (C. C. C., unpublished data). Regardless of the identity of the substrate interaction domain in Cdc4p, Skp1p most likely promotes substrate recruitment to SCF^{Cdc4p} either by forming direct contacts with bound substrate, or by evoking a conformational change in Cdc4p. The ability of Skp1p to enhance Cdc4p's substrate binding activity may prevent "free" Cdc4p from competing with SCF^{Cdc4p} for association with phosphorylated substrates. This notion is supported by our observation that recombinant Cdc4p does not competitively inhibit Sic1p ubiquitination in crude yeast extract, even when added in amounts 200-fold greater than those needed to achieve efficient complementation of the ubiquitination defect of *cdc4* mutant extract (Verma et al., 1997b; data not shown).

Whereas the phosphorylation of Sic1p triggers its ubiquitination by enhancing its association with Cdc4p/Skp1p, it remains unclear how Cdc4p/Skp1p discriminates between phosphorylated and unmodified Sic1p. We can envision two distinct models. Phosphorylation of Sic1p may cause a conformational rearrangement that exposes a cryptic Cdc4p/Skp1p-binding determinant in Sic1p. Alternatively, Cdc4p/Skp1p may bind directly to the phosphate groups on Sic1p, much like SH2 and 14-3-3 domains bind directly to phospho–amino acid epitopes (Songyang et al., 1993; Muslin et al., 1996). Mutagenesis experiments have shown that no single phosphorylation site on Sic1p is necessary or sufficient to specify its ubiquitination (Verma et al., 1997c). Instead, the mutagenesis data suggest that there is a set of key phosphorylation sites on Sic1p and that several distinct Sic1p phospho-isomers that are modified in a subset of these sites are competent to serve as substrates for SCF^{Cdc4p}-dependent ubiquitination. These data lead us to favor the idea that Cdc4p/Skp1p interacts directly with phosphorylated serine or threonine. Perhaps selective interaction with phosphorylated targets will prove to be a general property of proteins containing WD-40 repeats.

Willems et al. (1996) argued that Cdc53p selectively targets phosphorylated Cln2p for Cdc34p-dependent ubiquitination. However, these authors failed to demonstrate that the interaction they observed between Cdc53p and phospho-Cln2p was direct and not promoted by intermediary proteins. Our data suggest that this interaction was actually sustained by either Cdc4p or Grr1p (see below). Alternatively, Cdc53p may directly bind phosphorylated Cln2p, but this interaction may serve to recruit Cln2p/Cdc28p kinase activity to SCF, rather than to target Cln2p for ubiquitination.

Conservation and Generality of the SCF pathway

Phosphorylation triggers the degradation of a broad range of key regulatory proteins in metazoans, including G1 cyclins (Clurman et al., 1996; Won et al., 1996; Diehl et al., 1997); IκB, which is a global negative regulator of the immune response (Verma et al., 1995); CACTUS, which is required for dorsal-ventral patterning in Drosophila embryos (Belvin and Anderson, 1996); and B-catenin, which is involved in developmental patterning and acts as a tumor suppressor gene (Aberle et al., 1997). For IκB, CACTUS, and B-catenin, it is thought that phosphorylation-triggered degradation operates as a switch that governs the ultimate biological response. Based on our observations with Sic1p (Verma et al., 1997c; this paper), we speculate that regulated protein kinases and SCF-like complexes coordinately regulate diverse biological processes including the immune response, organismal development, and cell proliferation/tumor progression. Based on the known diversity of protein kinases and the potential diversity of SCF pathways (see below), an attractive model is that the ubiquitination of different SCF substrates is controlled by distinct, independently regulated kinases, or by multiple kinases acting in a combinatorial fashion (King et al., 1996).

Given the diverse range of substrates whose degradation is regulated by phosphorylation, a key question is, are there different SCF pathways in eukaryotic cells? Whereas Sic1p degradation requires Cdc34p, Cdc53p, Skp1p, and Cdc4p, Cln2p degradation requires Cdc34p, Cdc53p, Skp1p, and Grr1p (Barral et al., 1995; Deshaies et al., 1995; Bai et al., 1996; Willems et al., 1996), but not Cdc4p (R. J. D., unpublished data). Both Cdc4p and Grr1p contain an F box, which is a conserved domain that is thought to mediate binding to Skp1p (Bai et al., 1996). Thus, Cdc4p and Grr1p might serve as interchangeable modules that interact with a core ubiquitination machinery comprised of Skp1p, Cdc53p, and Cdc34p (Bai et al., 1996). This would allow for the assembly of two different ubiquitin ligase complexes with distinct substrate preferences; SCF^{Cdc4p} would target Sic1p for ubiquitination, whereas SCF^{Grr1p} would target Cln2p. We envision that the F box–containing receptor module would be recruited from a pool of inactive receptor subunits, only to be activated upon docking with Skp1p. In light of this model, it is perhaps surprising that Grr1p and Cdc4p share no homology outside of the F-box, even though Cln2p, like Sic1p, contains PEST sequences and must be phosphorylated to be recognized as a substrate for degradation (Lanker et al., 1996).

Besides Grr1p and Cdc4p, at least 30 F box–containing proteins have been detected in DNA sequence databases (Bai et al., 1996). Coupled with the diversity of

Cdc53p/cullin- and Skp1p-like proteins in humans, the broad pallette of F box–containing proteins would allow for the assembly of an array of SCFs with distinct substrate-binding specificities and modes of regulation. Thus, SCF-based ubiquitination pathways may play a broad role in eukaryotic regulatory biology.

Experimental Procedures

Insect Cells, Yeast Strains, and Reagents

Bacterial expression vectors for K48R-Ub and Cdc34p originated from J. Callis (University of California, Davis) and V. Chau (Wayne State University, Detroit, MI), respectively. The mouse monoclonal cell line expressing anti-polyoma antibodies was a generous gift from M. Peter (Swiss Institute for Experimental Cancer Research, Epalinges). A bacterial expression vector for GST-Skp1p, along with baculovirus constructs that express Skp1p, Skp1p^{HA}, or Skp1p^{His6}, were all generous gifts of P. Sorger (Massachusetts Institute of Technology, Cambridge). *CDC4*, *CDC53*, and SIC1^{HAHis6}-*T2A, T5G, T33A, S76A* plasmids were obtained from B. Jensen (University of Washington, Seattle), M. Goebl (Indiana University, Indianapolis), and R. Verma (this laboratory), respectively. Baculoviruses that express GST-Cdc28p^{HA} (J. W. Harper, Baylor College of Medicine, Houston, TX), Clb5p (M. Weinreich, Cold Spring Harbor), Cln2p, and Cdc28p^{HA} (Greg Reynard, this laboratory) were kindly provided by the indicated investigators.

Baculovirus Construction

Baculoviruses expressing Cln2p and Cdc28p^{HA} are described elsewhere (Reynard and Deshaies, unpublished data). The construction of a baculovirus encoding Cdc4p has been described previously (Verma et al., 1997b). Epitope-tagged versions of both *CDC4* and *CDC53* were constructed via insertion of a DNA cassette that contains two tandem repeats of a peptide epitope (MEYMPME) derived from the polyoma virus middle T protein (Grussenmeyer et al., 1985) followed by three tandem repeats of the hemagglutinin epitope (Field et al., 1988). This bipartite epitope is hereafter designated PH. An epitope-tagged version of Cdc4p was constructed by introducing via PCR a single copy of the PH cassette immediately after the start codon of Cdc4p present in pVL1392-CDC4 (Verma et al., 1997b).

To generate viral vectors for expression of Cdc53p, a portion of *CDC53* lacking its 60 C-terminal residues was amplified by PCR from pGEM53-8 (Mathias et al., 1996) and the resulting PCR product subcloned into pVL1392 (Pharmingen), yielding the vector pVL-53ΔC. A fragment containing the last 60 codons of *CDC53* was amplified by PCR from pGEM53-8 and subcloned into pVL-53ΔC to yield pVL-53, which contains the full-length open reading frame of *CDC53*. To generate an epitope-tagged version of Cdc53p, a fragment encoding the C-terminal 95 residues of Cdc53p was amplified by PCR and engineered to contain the PH cassette immediately upstream of the termination codon. This epitope-tagged version of CDC53 was subcloned into the vector pFastbac (GIBCO-BRL), yielding pFBCdc53p^{PH}. pVL-53, pVL-53ΔC and pFBCdc53p^{PH} were used to generate baculovirus stocks, per the manufacturers' instructions. All portions of *CDC4* and *CDC53* plasmids generated by PCR were sequenced.

Antibodies

Anti-Cdc53p antibodies were generated in rabbits immunized with an MBP fusion protein containing the last 23 residues of Cdc53p. Antibodies against MBP and Cdc53p were sequentially purified by affinity chromatography using antigen essentially as described (Harlow and Lane, 1988). Monoclonal anti-polyoma antibodies were purified out of tissue culture supernatant using protein A–Sepharose beads. Purified anti-polyoma antibodies were cross-linked to protein A with dimethylpimilimadate (Harlow and Lane, 1988) at a concentration of 2 mg of antibodies/mL of protein A resin.

Baculovirus Expression

Sf9 cells were infected with the appropriate baculovirus at an MOI of 10. Forty-two hours post-infection, cells were collected and lysed by resuspension in 1 ml of Buffer A per 10^7 cells (Buffer A: 20 mM HEPES [pH 7.4], 100 mM NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). Lysates were centrifuged at 14,000 × g for 15 min, and the supernatant (typically 5 mg of protein/ml) was adjusted to 10% glycerol, frozen in liquid nitrogen, and stored at −80°C.

^{PH}Cdc4p and Cdc53p^{PH} were affinity-purified as follows: 25 μl of anti-polyoma protein A beads was added to 500 μL of lysate and incubated at 4°C for 1 hr. The beads were then washed twice with 1 mL of Buffer A fortified with 0.2 M KOAc, and then twice with 1 mL of Buffer B (30 mM HEPES [pH 7.4], 300 mM KOAc, 0.1% octylglucoside, 10% glycerol, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 20 μg/mL Arg-insulin, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). Bound polyoma-tagged proteins were specifically eluted by incubation of the anti-polyoma beads in 50 μL of Buffer B containing 100 μg/mL polyoma peptide (MEYMPME) for 2 hr at 4°C.

Clb5p/Cdc28p^{HA}/Cks1p complexes were generated by coinfecting Sf9 cells with viruses encoding Clb5p and Cdc28p^{HA}. Cell lysates derived from these infections were incubated with Cks1p and immunopurified exactly as described for Cln2p/Cdc28p^{HA}/Cks1p complexes. Cln2p/GST-Cdc28p^{HA} kinase expressed in insect cells was purified by incubation of 200 μL of crude lysate with 25 μL of glutathione agarose beads for 30 minutes at 4°C. The beads were washed twice with the above lysis buffer and twice with Kinase Assay Buffer (KAB: 10 mM HEPES [pH 7.2], 50 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). The kinase complex either was left immobilized on the beads, thereby allowing for the subsequent removal of the complex after a kinase reaction, or was eluted by incubation of the resin in 30 μL of KAB containing 5 mM glutathione for 30 min at 4°C.

To purify a hexahistidine-tagged version of Skp1p (Skp1p^{His6}), Sf9 cells infected with a Skp1p-expressing baculovirus were harvested and lysed in Buffer C (25 mM HEPES [pH 7.4], 100 mM KCl, 10 mM imidazole, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin) supplemented with 50 mM NaF, 50 mM β-glycerophosphate, and 0.1% Triton X-100. After centrifugation (15 min, 14,000 × g), Skp1p^{His6} was adsorbed from the lysate onto Ni^+-NTA resin (Qiagen) and eluted in Buffer C plus 120 mM imidazole.

Bacterial/Yeast Expression and Purification of Proteins

Cdc34p was expressed and purified from E. coli as described previously (Banerjee et al., 1993). MBP-Sic1p^{MH6} was expressed and radiolabeled in E. coli and purified by successive steps of Ni^+-NTA and amylose affinity chromatography as described (Verma et al., 1997a). Other fusion proteins containing either maltose binding protein (MBP; New England BioLabs) or glutathione-S transferase (GST; Pharmacia) moieties were expressed and purified as specified by the supplier, and stored in 25 mM HEPES [pH 7.2], 100 mM NaCl, 2 mM DTT, 1 mM EDTA, and 10% glycerol at −80°C.

Saccharomyces cerevisiae Uba1p was purified from the yeast strain JD77 (Dohmen et al., 1995) transformed with the plasmid pJD325 (J. Dohmen, personal communication), which carries a copper-inducible promoter driving expression of hexahistidine-tagged Uba1p (^{His6}Uba1p). Log-phase JD77[pJD325] cells grown in 1L YPD were induced with $CuSO_4$ to 1 mM, harvested 24 hr after induction, washed, and frozen in liquid nitrogen. The frozen cell mass was ground with a chilled mortar and pestle, and the resulting powder was resuspended in Buffer D (50 mM Tris [pH 7.5], 0.1% Triton X-100, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin) and then clarified by centrifugation (30 min, 35,000 × g). ^{His6}Uba1p was purified from the lysate by Ni^+-NTA affinity chromatography followed by ubiquitin-affinity chromatography (Ciechanover et al., 1982). Purified ^{His6}Uba1p was dialyzed twice against 1 L of 50 mM Tris [pH 7.6], 0.1 M KCl, 0.2 mM DTT, 15% glycerol and then adjusted to 0.2 mg/mL BSA and stored in aliquots at −80°C.

Ubiquitination Reactions

All ubiquitination reactions contained: 4 μg ubiquitin, 60 ng Cdc34p, 25 ng ^{His6}Uba1p, 1 μL of a 10× ATP-regenerating system (20 mM HEPES [pH 7.2], 10 mM ATP, 10 mM MgOAc, 300 mM creatinine phosphate, 0.5 mg/mL creatinine phosphokinase), 1 μL of 10× reaction buffer (40 mM MgOAc, 10 mM DTT, 1 mM PMSF), and ~10,000

cpm [^{35}S]-MBP-Sic1p^{MH6} (~20 ng). Reactions performed with crude lysates included: 1 μL each of Sf9 cell lysate containing Cdc4p, Cdc53p, or Skp1p; and 2 μl of affinity-purified Cln2p/Cdc28p^{HA}/Cks1p kinase. Reactions utilizing pure protein components contained 25 ng of either GST-Skp1p or Skp1p^{His6}, plus 0.5 μL each of eluted ^{PH}Cdc4p and Cdc53p^{PH} (from a 50 μL immunopurification as described above). For the experiments shown in Figure 3B, wild-type ubiquitin was replaced with 1.5 μg of either methyl ubiquitin or K48R ubiquitin. Reactions using MBP-Cdc4p contained 9 μg of the preparation shown in Figure 2A. Each ubiquitination component was present at saturating levels relative to substrate (data not shown). Reactions were brought to a final volume of 10 μL with 20 mM HEPES [pH 7.4], 100 mM KOAc, 1 mM DTT. All components were mixed and incubated at 25°C for 90 minutes (unless otherwise indicated). Reactions were terminated by addition of Laemmeli sample buffer, resolved by SDS–PAGE, and visualized by autoradiography.

To phosphorylate [^{35}S]-MBP-Sic1p^{MH6}, 85 μL of purified radiolabeled substrate (approximately 1 μg) was mixed with 8 μL of glutathione-agarose-bound Cln2p/GST-Cdc28p^{HA}/Cks1p, and the reactions were diluted to a final volume of 100 μL with KAB and then adjusted to 1 mM ATP. The mixture was gently rocked at room temperature for 2 hr, after which the beads were pelleted by centrifugation (5 sec, 13,000 × g) and the supernatant was used in reactions as described.

Immunoprecipitations

At 40 hr post-infection, baculovirus-infected Sf9 cells were metabolically labeled for 3 hr in methionine-deficient media by the addition of Tran[^{35}S]-label (10 μCi/mL), and then lysates were prepared as described above. Coimmunoprecipitation experiments with MBP-Sic1p^{MH6} were carried out by combining 25 μL of the appropriate Sf9 cell lysate with 1 μg of either unphosphorylated or phosphorylated MBP-Sic1p^{MH6} plus sufficient Buffer A to yield a final volume of 100 μl. The mixtures were incubated for 1 hr at 4°C and immunoprecipitated by addition of anti-MBP antibodies Following a final 1 hr incubation at 4°C, immunoprecipitates were collected by centrifugation and washed with Buffer A containing 5 mM NaF. The resulting samples were analyzed by SDS–PAGE and autoradiography.

PCR templates of Sic1p carrying a tandem hemagglutinin epitope plus hexahistidine (HAHis6) tag at its C-terminus (Sic1p^{HAHis6}), and a mutant form of Sic1p^{HAHis6} (Sic1p-T5G, T33A, S76A; Sic1p^{HAHis6}-Δ3P) were translated in rabbit reticulocyte lysate to produce [^{35}S]-labeled proteins (Verma et al., 1997c). Phosphorylation of both constructs by Cln2p/Cdc28p^{HA}/Cks1p was done essentially as described for [^{35}S]-MBP-Sic1p^{MH6}. To produce samples that were not phosphorylated, substrates were incubated with immobilized kinase in the absence of exogenously added ATP. To test the ability of these proteins to bind ^{PH}Cdc4p, 1 mL of insect cell lysate containing ^{PH}Cdc4p was incubated with 30 μL of anti-polyoma resin for 1 hr at 4°C and then washed three times with Buffer A. Cdc4p:Sic1p binding reactions were carried out in a final volume of 100 μL and contained 10 μL of a 16% slurry of ^{PH}Cdc4p-containing beads, 15 μL of protein A beads (pretreated with 1% bovine serum albumin), and either 3 μL ([^{35}S]-MBP-Sic1p^{MH6}) or 5 μL ([^{35}S]-Sic1p^{HAHis6} and [^{35}S]-Sic1p^{HAHis6}-Δ3P) of ligand. Following a 1 hr incubation at 4°C, the beads were washed three times with Buffer A containing 5 mM NaF, boiled in Laemmeli sample buffer, and subjected to SDS–PAGE and autoradiography.

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