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F-Box Proteins Are Receptors that Recruit Phosphorylated Substrates to the SCF Ubiquitin-Ligase Complex

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Summary

We have reconstituted the ubiquitination pathway for the Cdk inhibitor Sic1 using recombinant proteins. Skp1, Cdc53, and the F-box protein Cdc4 form a complex, SCF^{Cdc4}, which functions as a Sic1 ubiquitin-ligase (E3) in combination with the ubiquitin conjugating enzyme (E2) Cdc34 and E1. Cdc4 assembled with Skp1 functions as the receptor that selectively binds phosphorylated Sic1. Grr1, an F-box protein involved in Cln destruction, forms complexes with Skp1 and Cdc53 and binds phosphorylated Cln1 and Cln2, but not Sic1. Because the constituents of the SCF complex are members of protein families, SCF^{Cdc4} is likely to serve as the prototype for a large class of E3s formed by combinatorial interactions of related family members. SCF complexes couple protein kinase signaling pathways to the control of protein abundance.

Introduction

Protein degradation is a commonly employed mechanism for the control of protein abundance. It is a particularly effective method for promoting unidirectional cell cycle transitions because of its rapidity and irreversibility. Three major transitions, entry into S phase, separation of sister chromatids, and exit from mitosis, require the degradation of specific proteins via the ubiquitin-26S proteasome pathway (reviewed in King et al., 1996). The formation of ubiquitin-protein conjugates involves three components that participate in a cascade of ubiquitin transfer reactions: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a specificity factor (E3) (Hershko et al., 1983). Ubiquitin is activated as a thiol-ester on E1 in an ATP-dependent reaction, transferred to an E2 as a thiol ester, and ultimately conjugated to the target protein in conjunction with an E3, which functions in substrate recognition and in some instances may serve as a thiol-ubiquitin carrier (Scheffner et al., 1995). Together, these enzymes polyubiquitinate lysine residues in target proteins through formation of isopeptide bonds with ubiquitin, leading to recognition by the 26S proteasome.

E1 and E2 proteins can be identified through sequence similarity, but this is not generally true for E3s. The identity of E3s are a central issue because they are potential regulators of ubiquitination timing and substrate selection. Much of our current knowledge of E3s comes from analysis of the HECT domain protein E6-AP which functions as a ubiquitin-ligase for p53, (Huibregtse et al., 1995; Scheffner et al., 1995), and the anaphase-promoting complex (APC), which functions in mitotic cyclin destruction and sister chromatid cohesion (King et al., 1996). Although most APC substrates contain a destruction box motif, precisely how the timing and selection of substrates by the APC is achieved is unknown. In contrast, timing of ubiquitination of a variety of non-APC substrates is thought to be regulated in part by the phosphorylation of the substrate itself. PEST sequences (rich in proline, glutamic acid, serine, and threonine) are frequently found in unstable proteins such as cyclins and contain sites of phosphorylation. Phosphorylation of specific residues has been implicated in the destruction of G1 cyclins (Tyers et al., 1992; Clurman et al., 1996; Lanker et al., 1996; Won and Reed, 1996; Diehl et al., 1997), and the cyclin-kinase inhibitor (CKI) p27 (Sheaff et al., 1997).

In Saccharomyces cerevisiae, entry into S phase requires activation of the Cdc28 kinase by G1 cyclins (Cln1, Cln2, and Cln3) and S-phase cyclins (Clb5 and Clb6). Although both Cln/Cdc28 and Clb/Cdc28 complexes assemble during G1, Clb/Cdc28 is inactive through association with the CKI p40^{Sic1} (Mendenhall, 1993; Schwob et al., 1994). Sic1 abundance sharply decrease at the G1/S transition, correlating with Clb5/Cdc28 activation. The decrease in Sic1 levels depends on the E2 Cdc34, implying that ubiquitination triggers Sic1 destruction (Schwob et al., 1994). Sic1 destruction also requires *CLN* and *CDC28* function, and elimination of Sic1 is the primary role for Cln/Cdc28 activity in S-phase entry (Schneider et al., 1996; Schwob et al., 1994; Tyers, 1996). Although Sic1 is a phosphoprotein (Schneider et al., 1996), it is not known whether Cln/Cdc28 complexes directly phosphorylate Sic1 or whether phosphorylation plays another, perhaps indirect, role in Sic1 destruction.

Three other genes, *SKP1*, *CDC53*, and *CDC4*, are required for S-phase entry (Schwob et al., 1994; Bai et al., 1996). These genes, together with *CDC34*, show a pattern of suppression and enhancement consistent with roles in a common process and conditional alleles of these genes cause arrest with unreplicated DNA and multiple buds (Yochem and Byers, 1987; Goebl et al., 1988; Bai et al., 1996; Mathias et al., 1996). Sic1 accumulates in *cdc34-1*, *cdc4-1*, or *skp1-11* mutants and deletion of *SIC1* allows such mutants to undergo DNA synthesis (Bai et al., 1996; Schwob et al., 1994). Components of the Cdc34 pathway have also been implicated in the

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destruction of a number of other important regulatory proteins, including yeast G1 cyclins (Deshaies et al., 1995; Yaglom et al., 1995; Bai et al., 1996; Willems et al., 1996), Cdc6 (Piatti et al., 1996), the CKIs Rum1 and Far1 (McKinney et al., 1993; Henchoz et al., 1997; Kominami and Toda, 1997), and the transcription factor Gcn4 (Kornitzer et al., 1994). Thus, Cdc34, Cdc53, Skp1, and Cdc4 may be utilized for the destruction of diverse regulatory proteins. A requirement for Cdc34 for Cln2 ubiquitination has been demonstrated in crude yeast extracts (Deshaies et al., 1995), but this requirement has been suggested to be indirect (Blondel and Mann, 1996). Interestingly, *SKP1* is also required for the G2/M transition (Bai et al., 1996; Connelly and Hieter, 1996) and has been found to be a component of the kinetochore complex CBF3 (Connelly and Hieter, 1996; Stemmann and Lechner, 1996). The function of Skp1 in the kinetochore is unknown.

Skp1 binds the F-box motif in Cdc4 (Bai et al., 1996). F boxes are found in many Skp1-interacting proteins including cyclin F (Bai et al., 1994) and the cyclin A/Cdk2-associated protein Skp2 (Zhang et al., 1995). The two largest classes of F-box proteins either contain WD-40 repeats like Cdc4 or leucine-rich repeats (LRR) like Skp2 and Grr1 (Bai et al., 1996). *GRR1* was initially identified as a gene required for glucose repression (Flick and Johnston, 1991) but was later found to be involved in Cln destruction (Barral et al., 1995). The fact that Skp1 is required for the destruction of both Sic1 and Cln2 while Cdc4 and Grr1 had only been implicated in the destruction of one of these led us to propose the model in which F-box proteins recognize ubiquitination targets and that Skp1 links these F-box/target complexes to the ubiquitination machinery (Bai et al., 1996).

In this study, we explored the role of Skp1 and F-box proteins in ubiquitination through in vitro reconstruction of the Sic1 ubiquitination pathway. Sic1 ubiquitination requires all of the components implicated by genetic analysis. Skp1 functions to recruit Cdc4 into a Cdc53/Cdc34 complex and enhances recognition of Sic1 by Cdc4, the later interaction requiring Sic1 phosphorylation. In contrast, Grr1 does not interact with Sic1 but does recruit phosphorylated Cln1 and Cln2 into Skp1/Cdc53 complexes. Our results indicate that F-box proteins function as receptors that recruit substrates into a Skp1/Cdc53/Cdc34 complex for ubiquitination.

Results

Assembly of a Complex Containing Cdc53/Skp1/Cdc4 and the E2 Cdc34

Cdc34, Cdc53, Skp1, and Cdc4 have been genetically implicated in control of Sic1 destruction, although the functions of these proteins in this process are not known. We previously showed that *SKP1* and *CDC4* show reciprocal overproduction suppression of their respective temperature-sensitive mutants and that their encoded proteins physically interact (Bai et al., 1996). A further search for suppressors using a *GAL*-driven cDNA library revealed that *CDC53* overexpression suppresses *skp1-11* (data not shown). These findings, coupled with genetic and physical evidence of a Cdc53/Cdc34 interaction (Mathias et al., 1996; Willems et al., 1996), led us to examine whether Cdc53 and Skp1 interact and whether Skp1 may facilitate interaction of Cdc53 with Cdc4 and/or Cdc34.

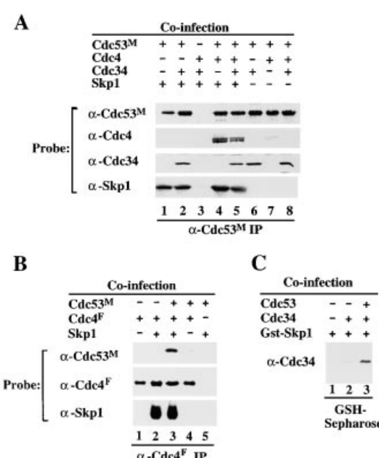

Figure 1. Assembly of a Multiprotein Complex Containing Cdc34, Cdc53, Skp1, and Cdc4

(A and B) Formation of a Cdc53/Cdc4 complex is enhanced by Skp1. Whole cell lysates were derived from 4×10^5 insect cells infected with the indicated combinations of baculoviruses. Complexes were then immunoprecipitated via either (A) a Myc tag on Cdc53 ($Cdc53^M$) using anti-Myc antibodies or (B) a Flag tag on Cdc4 ($Cdc4^F$). Immune complexes were immunoblotted and probed with anti-Myc to detect $Cdc53^M$, anti-Cdc4, anti-Cdc34, and anti-Skp1.

(C) Skp1 and Cdc34 can associate with Cdc53 simultaneously. Gst-Skp1 complexes were purified from insect cells infected with the indicated baculoviruses using GSH-Sepharose prior to SDS–PAGE and immunoblotting with anti-Cdc34 antibodies.

Insect cells were coinfected with various combinations of baculoviruses expressing Myc-tagged Cdc53 ($Cdc53^M$), Cdc34, Cdc4, and Skp1, and anti-Myc immune complexes from the indicated lysates were immunoblotted for associated proteins (Figure 1A). In the presence of all four proteins, anti-$Cdc53^M$ complexes contain Cdc4, Cdc34, and Skp1 (Figure 1A, lane 5). However, in the absence of Skp1, only low levels of Cdc4 bound $Cdc53^M$, regardless of the presence of Cdc34 (Figure 1A, lanes 7 and 8). This result was confirmed through the analysis of $Cdc53^M$ association with anti-$Cdc4^F$ immune complexes (Figure 1B). Thus, Skp1 facilitates association of Cdc53 with Cdc4. In contrast, both Skp1 and Cdc34 can simultaneously associate with $Cdc53^M$ in the absence of other yeast proteins (Figures 1A and 1C). Taken together, these data indicate that Cdc34, Cdc53, Skp1, and Cdc4 form a multiprotein complex.

Phosphorylation of Sic1 by Cln/Cdc28 Is Required for Its Recognition by a Cdc53/Skp1/Cdc4 Complex

Previous studies have implicated Cln/Cdc28 kinase activity in Sic1 degradation (Schwob et al., 1994; Schneider et al., 1996; Tyers, 1996). However, it was not clear: (1) whether Sic1 was directly phosphorylated by Cln/Cdc28, (2) whether this phosphorylation was correlative or causative for subsequent Sic1 degradation, (3) if causative, whether this modification played a role in

Sic1 recognition by the ubiquitination machinery, and (4) whether Cln/Cdc28 might also regulate the activity of the ubiquitination machinery directly. To address these questions, we purified Cln1/Gst-Cdc28 and Sic1/Clb5/Gst-Cdc28 complexes from insect cells (Figures 2A and 2B). In principle, Sic1/Clb/Cdc28 complexes should represent the primary form of Sic1 ubiquitinated in vivo. To circumvent Sic1 phosphorylation by excess Clb5/Cdc28, we used a kinase-impaired Cdc28(K−) (D154N). Sic1 in such complexes remains essentially unphosphorylated unless treated with Cln1/Cdc28 (Figure 2B). In vitro phosphorylation of Sic1 reduced its electrophoretic mobility as has been observed in vivo (Schwob et al., 1994).

Phosphorylated and unphosphorylated Sic1 were used in binding reactions with anti-Cdc53^{M} immune complexes assembled and purified from insect cells (Figure 2C). Phosphorylated Sic1 efficiently associates with Cdc53/Skp1/Cdc4 complexes, and this association depends upon the presence of Skp1 (Figure 2C, lanes 6 and 8). This suggest that Cdc4 and/or Skp1 function as binding factors for Sic1. Typically 10%–20% of the input phosphorylated Sic1 was bound at about 20 nM Sic1. In contrast, the extent of binding of unphosphorylated Sic1 (<1%, lane 7) was comparable to that observed in control immune complexes generated from uninfected cells (lane 3). Thus, association of Sic1 with the Cdc53/Skp1/Cdc4 complex requires phosphorylation by Cln1/Cdc28.

Cdc4 Is the Specificity Factor for Recognition of Phosphorylated Sic1

To directly examine the roles of Skp1 and Cdc4 in Sic1 recognition, binding experiments were performed using a series of complexes assembled in vivo that contained constant high levels of Flag-tagged Skp1 (Skp1^{F}) and increasing quantities of Cdc4. Association of phosphorylated Sic1 with anti-Skp1^{F} immune complexes was absolutely dependent upon the presence of Cdc4 (Figure 2E, compare lanes 3 and 9). Moreover, deleting the last three WD-40 repeats from the C terminus of Cdc4 (Cdc4ΔWD) abolished its ability to bind phosphorylated Sic1 (Figure 2E, lanes 10–16). Therefore, Cdc4 functions as the specificity factor for binding of phosphorylated Sic1, and the Cdc4/Sic1 interaction requires an intact WD-40 repeat domain in Cdc4. While Skp1 alone does not interact with Sic1, it does stimulate association of Sic1 with Flag-Cdc4 (Cdc4^{F}) by about 5-fold (Figure 2D). It is not clear if Skp1 physically contacts Sic1 or stabilizes a form of Cdc4 compatible with Sic1 binding, nor is it clear whether the weak association of Sic1 with

Figure 2. A Skp1/Cdc4 Complex Functions in Recognition of Phosphorylated Sic1

(A) SDS–PAGE analysis of Cln1^{HA}/Gst-Cdc28^{HA}/Cks1 complexes purified from insect cells. Asterisk indicates position of endogenous GST protein.

(B) Phosphorylation of Sic1 by Cln1/Cdc28 in vitro. Sic1/Clb5/Gst-Cdc28^{HA}(K−) (1 μM) was incubated for 45 min at 25°C with 500 μM ATP (lanes 1) or 50 nM Cln1/Cdc28 and 500 μM ATP (lane 2), resulting in reduced electrophoretic mobility for Sic1. In lanes 4 and 5, smaller amounts of Sic1 phosphorylation reactions performed in the presence of γ-^{32}P-ATP were separated by SDS–PAGE prior to autoradiography. Lane 3 shows the reaction products obtained when Cln1/Cdc28 complexes alone were incubated with γ-^{32}P-ATP.

(C) Phosphorylation of Sic1 is required for its association with Cdc34/Cdc53/Skp1/Cdc4 complexes. Anti-Cdc53^{M} immune complexes derived from insect cells infected with the indicated baculoviruses were used in a binding assay with Cln1/Cdc28-phosphorylated or unphosphorylated Sic1/Clb5/Cdc28 and bound Sic1 detected by immunoblotting with anti-Sic1 antibodies.

(D) Association of phosphorylated Sic1 with Cdc4 is enhanced by Skp1. Anti-Flag immune complexes derived from cells infected with constant high quantities of Cdc4^{F} virus (lanes 3–9) and increasing quantities of Skp1 virus (lanes 4–10) were used in binding assays with Cln1/Cdc28-phosphorylated Sic1. Bound proteins were separated by SDS–PAGE and immunoblots probed with the indicated antibodies.

(E) Association of phosphorylated Sic1 with Skp1 requires the WD-40 repeats of Cdc4. Insect cells were coinfected with constant quantities of Skp1^{F} virus and increasing quantities of Cdc4 (lanes 4–9) or Cdc4ΔWD virus (lanes 12–17). Anti-Skp1^{F} immune complexes were used in binding assays with phosphorylated Sic1 and bound proteins detected as in (C).

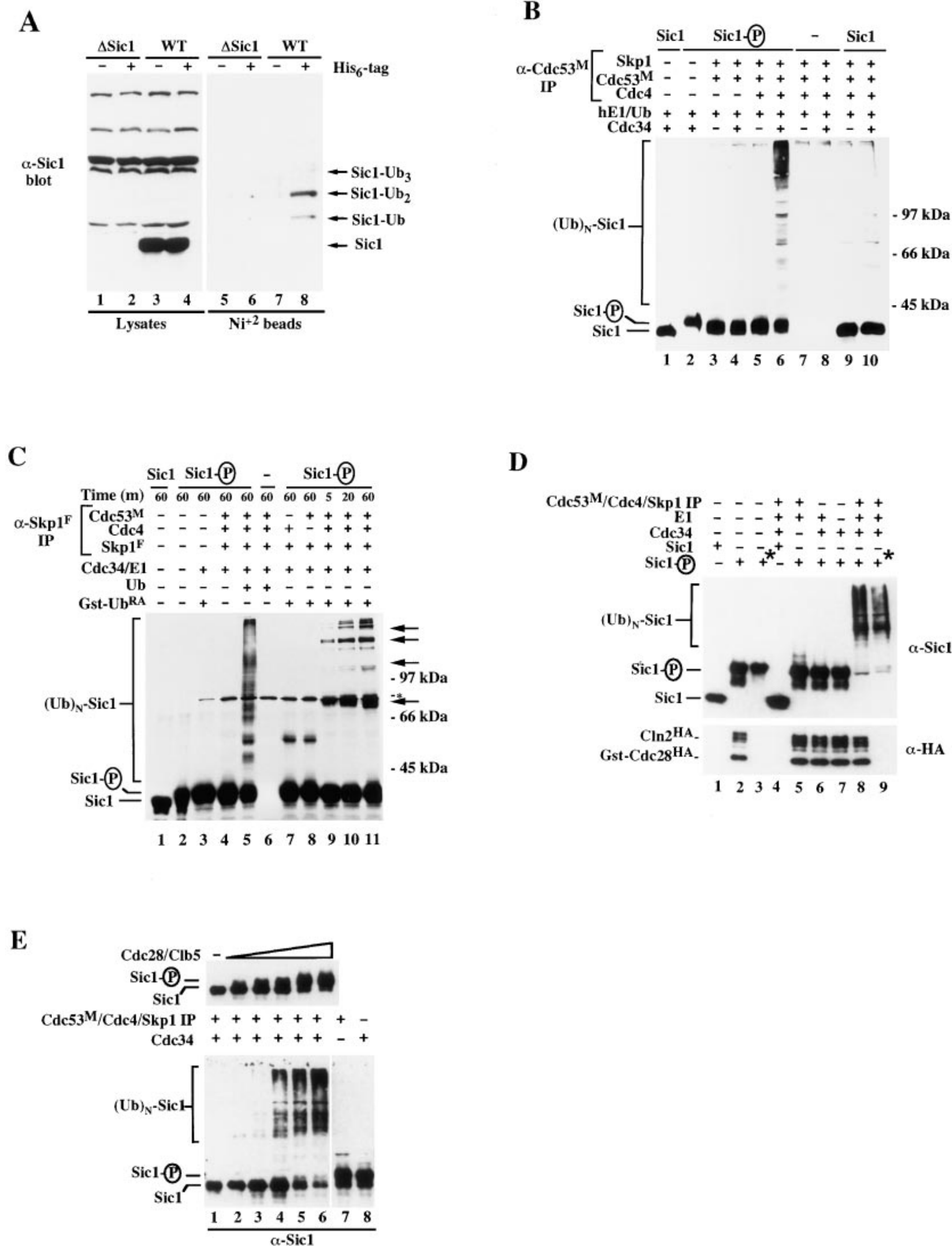

Figure 3. Sic1 Is Ubiquitinated In Vivo and Can Be Ubiquitinated In Vitro by a Cdc53/Skp1/Cdc4 Complex Together with Cdc34

(A) Ubiquitination of Sic1 in vivo. Extracts from wild-type or *sic1* deletion strains expressing either *pCUP1-UBI*^{RA} or *pCUP1-UBI*$^{HIS\text{-}MYC\text{-}RA}$ were incubated with Ni^{+2}-NTA beads, and bound proteins separated by SDS–PAGE and immunoblotted with anti-Sic1 sera. The position of Sic1 and Sic1-ubiquitin conjugates are indicated.

(B) Anti-Cdc53^{M} immune complexes were prepared from insect cells expressing Cdc53^{M}/Skp1 (lanes 3 and 4) or Cdc53^{M}/Skp1/Cdc4 (lanes 5–10) and supplemented with ATP, ubiquitin (lanes 1–10), human E1 (lanes 1–10), Cdc34 purified from E. coli (lanes 1, 2, 4, 6, 8, and 10), and either unphosphorylated (lanes 1, 9, and 10) or phosphorylated Sic1/Clb5/Cdc28 (lanes 2–6). After 60 min at 25°C, proteins were separated by SDS–PAGE and immunoblotted using anti-Sic1 antibodies.

(C) Anti-Skp1^{F} immune complexes were generated from insect cells infected with either Skp1^{F}/Cdc53^{M}/Cdc4 (lanes 4–6, and 9–11), Skp1^{F}/Cdc4 (lane 7), or Skp1^{F}/Cdc53^{M} (lane 8) and reactions performed as in (B). Where indicated, Gst-Ub^{RA} replaced ubiquitin. The asterisk indicates an 80 kDa protein that cross-reacts with some anti-Sic1 antibodies. The arrows indicate Gst-Ub^{RA}-Sic1 conjugates.

(D) Ubiquitination of Sic1 does not require that Cln/Cdc28 be present in the ubiquitination reaction nor that Sic1 be associated with Clb5/Cdc28. Sic1 was purified from bacteria (lane 1) and treated with soluble (lane 2) or immobilized (lane 3) Cln2/Gst-Cdc28. Use of phosphorylated Sic1 that was free of Cln2 kinase (verified by immunoblotting with anti-HA antibodies) is indicated by an asterisk (lanes 3 and 9). Sic1 proteins were then used in ubiquitination reactions (30 min) with Skp1^{F}/Cdc53^{M}/Cdc4 complexes as in (B), except that yeast E1 replaced human E1.

(E) Clb5/Cdc28-phosphorylated Sic1 is a substrate for ubiquitination by Cdc34. Sic1 was treated with increasing Clb5/Cdc28 until the kinase was in excess as determined by histone kinase assays (data not shown). Under these conditions, Sic1 electrophoretic mobility is reduced (lanes 1–6, top). Aliquots of differentially phosphorylated Sic1 were used in ubiquitination reactions as in (D) (lanes 1–6). As a negative control, partially phosphorylated Sic1 corresponding to the Sic1 protein in lane 5 (top) was reacted in the absence of Cdc34 (lane 7) or the Cdc53^{M}/Cdc4/Skp1 complex (lane 8).

Cdc4 alone (Figure 2D, lane 3) reflects participation of an insect cell Skp1 homolog. Nevertheless, our results clearly demonstrate a positive contribution of Skp1 in the Cdc4/Sic1 interaction.

Sic1 Is Ubiquitinated In Vivo

While our finding that Cdc4, Skp1, and Cdc53 form a complex that binds both phosphorylated Sic1 and Cdc34 is consistent with a role for ubiquitination in the regulation of Sic1 abundance, to date, in vivo ubiquitination of Sic1 has not been demonstrated. To address this, we used Ni^{+2}-NTA resin to isolate ubiquitinated proteins from extracts of wild-type cells or *sic1* deletion mutants expressing His_6-Ub^{RA} or Ub^{RA} (Willems et al., 1996). The K48R mutation in Ub^{RA} blocks polyubiquitin chain formation and proteasome recognition (Chau et al., 1989). A ladder of bands recognizable by anti-Sic1 antibodies was detected in ubiquitin conjugates from wild-type lysates containing His_6-Ub^{RA} (Figure 3A, lane 8) but not in conjugates derived from Ub^{RA}-expressing cells or a *sic1* deletion strain (Figure 3A, lanes 5 and 6). Thus, Sic1 is ubiquitinated in vivo.

Reconstitution of the Sic1 Ubiquitination Pathway Using Recombinant Proteins

We examined whether Cdc53/Skp1/Cdc4 complexes can catalyze ubiquitination of Sic1 when supplemented with Cdc34, E1, ATP, and ubiquitin. In the presence of all reaction components, phosphorylated Sic1 in complexes with Clb5/Cdc28 was efficiently converted to higher molecular weight conjugates detectable with anti-Sic1 antibodies (Figure 3B, lane 6; Figure 3C, lane 5). Unphosphorylated Sic1 was not detectably ubiquitinated. Sic1 ubiquitination absolutely required Cdc34, Cdc4, Cdc53, E1, and ubiquitin (Figures 3B and 3C), as well as yeast Skp1 (data not shown). When Gst-Ub^{RA} was used, the reaction products formed over time were integrated into a ladder of bands differing by $\sim$35 kDa, the size of Gst-Ub^{RA} (Figure 3C, lane 11), indicating that the higher molecular weight forms of Sic1 are due to its ubiquitination. Since Gst-Ub^{RA} poorly forms polyubiquitin chains, the number of bands observed likely reflects the number of individual lysines ubiquitinated on a single Sic1 molecule. The association of Sic1 with Clb5/Cdc28 complexes was not required for its ubiquitination (Figure 3D). Sic1 purified from bacteria and phosphorylated by Cln2/Cdc28 was also efficiently ubiquitinated, with greater than 90% of the Sic1 forming ubiquitin conjugates (lane 8) while unphosphorylated Sic1 was not ubiquitinated (lane 4). We also found that greater than 50% of the Sic1-ubiquitin conjugates formed after 60 min had dissociated from the Cdc4/Skp1/Cdc53 complex (data not shown). In these reactions, neither Gst-Cdc28, Clb5, Cdc53, Skp1, or Cdc4 were ubiquitinated, although Cdc34 was ubiquitinated on lysine (data not shown).

Although phosphorylation of Sic1 was required for its recognition by Cdc4 and Skp1, it remained possible that active Cln/Cdc28 was also required for additional steps in the ubiquitination process. To perform the ubiquitination in the absence of Cln kinase, bacterial Sic1 was phosphorylated with Cln2/Gst-Cdc28 immobilized on GSH-Sepharose beads and removed from the beads prior to use in ubiquitination reactions (Figure 3D, lane 3). Sic1 phosphorylated in this manner was efficiently ubiquitinated (Figure 3D, lane 9), indicating that Sic1 phosphorylation constitutes the primary requirement of Cln/Cdc28 kinases in Sic1 ubiquitination in the in vitro reaction.

Although Sic1 is an inhibitor of Cdc28/Clb5 complexes, when the kinase complex was in excess, it is capable of phosphorylating Sic1 (Figure 3E) and converting it into a substrate for ubiquitination (Figure 3E). This result may explain the fact that overexpression of *CLB5* can drive S-phase entry in cln^- cells and suggests that active Clb5/Cdc28 formed during Sic1 destruction may collaborate with Cln/Cdc28 to accelerate the Sic1 ubiquitination process.

F-Box Proteins Are Receptors for Specific Ubiquitination Substrates

Our results are consistent with the hypothesis that F-box proteins can function in the recognition of ubiquitination targets. To explore the selectivity of F-box proteins, we tested whether substitution of Cdc4 by another F-box protein Grr1 could support Sic1 binding and ubiquitination. Gene10-tagged Grr1 ($Grr1^{G10}$) can interact simultaneously with Skp1 and Cdc53 when coexpressed in insect cells (Figure 4A) and Cdc53/Skp1 interacts with Grr1 and Cdc4 in a mutually exclusive manner (data not shown). Unlike Cdc4, the Grr1/Cdc53 interaction in insect cells was not enhanced by coexpression of Skp1 although Skp1 assembled with these complexes (data not shown). Importantly, Cdc53/Skp1/Grr1 complexes were unable to associate with phosphorylated Sic1 and did not support ubiquitination of phosphorylated Sic1 complexes (Figures 4B and 4C). Therefore, F-box proteins can display selectivity toward particular targets.

Grr1 Binds Cln1 and Cln2 in a Phosphorylation-Dependent Manner

The observation that Cdc4 recognizes Sic1, together with the genetic requirement for Grr1 in Cln destruction, suggested that Grr1 might function in Cln recognition. In addition, since mutation of potential Cdc28 phosphorylation sites in the PEST domain in Cln2 increase its stability in vivo (Lanker et al., 1996) and only phosphorylated Cln2 associates with Cdc53 in vivo (Willems et al., 1996), it seemed likely that binding would require Cln phosphorylation. Therefore, we examined whether Grr1 could bind phosphorylated Clns. Cln1/Gst-Cdc28 and Cln2/Gst-Cdc28 complexes were isolated from insect cells and autophosphorylated by incubation with [γ-^{32}P]-ATP. This modification reduces their electrophoretic mobility (see below). These ^{32}P-labeled Cln complexes were then used in binding reactions with purified $Skp1^F$/Grr1 and $Skp1^F$/Cdc4 complexes containing comparable amounts of Grr1 and Cdc4 (Figure 5B and data not shown). Both Cln1 and Cln2 efficiently associated with $Skp1^F$/Grr1 complexes (Figure 5A, lanes 8 and 12) in the presence or absence of Cdc53 (lane 16). In contrast, Cln proteins associated more weakly with Cdc4/$Skp1^F$ complexes (lanes 7, 11, and 15), although the extent of binding was higher than in the absence of Cdc4 (lanes 6, 10, and 14). In contrast, Sic1 exclusively

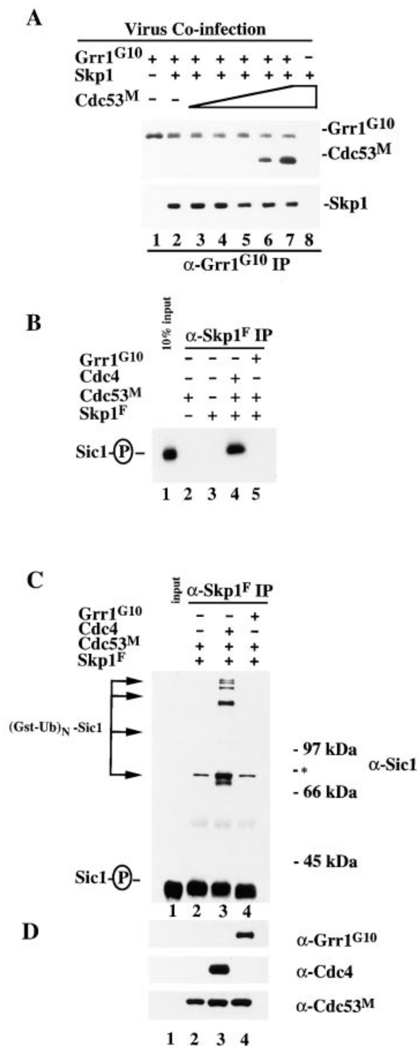

Figure 4. Grr1 Forms Complexes with Skp1 and Cdc53 but Cannot Substitute for Cdc4 in Recognition and Ubiquitination of Sic1

(A) Grr1 can associate with Skp1 and Cdc53. Grr1 complexes were immunoprecipitated from insect cell lysates using anti-G10 antibodies. Proteins were separated by SDS–PAGE and immunoblotted with anti-G10 and anti-Myc to detect $Grr1^{G10}$ and $Cdc53^{M}$, or with anti-Skp1 antibodies.

(B) Phosphorylated Sic1 associates with Cdc4 but not Grr1-containing complexes. Anti-$Skp1^{F}$ immune complexes derived from insect cells infected with the indicated baculovirus combinations (lanes 2–5) were used for binding reactions with ^{32}P-labeled Sic1 complexes. Bound Sic1 was visualized by SDS–PAGE and autoradiography. Ten percent of the input Sic1 complex (lane 1) was included as a control. The presence of Cdc4, Skp1, Cdc53, and Grr1 was verified by immunoblotting (data not shown).

(C) Cdc4, but not Grr1, supports ubiquitination of Sic1 in vitro. The indicated anti-$Skp1^{F}$ immune complexes were used in ubiquitination assays as described in the legend to Figure 3, employing $Gst\text{-}Ub^{RA}$ as the ubiquitin source.

(D) The presence of components was verified by reprobing blots with the indicated antibodies.

associated with Cdc4 complexes, demonstrating that Grr1 and Cdc4 display specificity toward physiological substrates.

To examine whether Cln1/Grr1 association was phosphorylation-dependent, Grr1 alone or in complexes with Skp1 or Skp1/Cdc53 was immunoprecipitated from insect cell lysates and used in binding assays with phosphorylated or unphosphorylated Cln1 complexes (Figure 5C). Unphosphorylated Cln1 was produced in insect cells as a complex with kinase-deficient Gst-Cdc28(K−), which minimized Cln1 autophosphorylation during expression and allowed the role of phosphorylation to be tested. Unphosphorylated Cln1 migrates at ∼66 kDa (Figure 5C, lane 1), while phosphorylated Cln1 (lane 2) migrates at 80 kDa. Both forms are observed in vivo (Lanker et al., 1996). Phosphorylated Cln1 (and its associated Cdc28 protein) efficiently associated with all Grr1 complexes (Figure 5C, lanes 6, 8, and 10) but was absent from reactions lacking Grr1 (Figure 5C, lane 4). In contrast, the amount of unphosphorylated Cln1 associated with Grr1 was comparable to that observed in binding reactions lacking Grr1 (Figure 5C, lanes 3, 5, 7, and 9). Thus, association of Cln1 with Grr1 is greatly enhanced by phosphorylation. Although the Grr1/Skp1/Cdc53 complex is capable of binding efficiently to phosphorylated Cln1, it was not competent for Cln1 ubiquitination when supplemented with Cdc34 and E1 (Figure 5D). Moreover, Cdc4 complexes that function in Sic1 ubiquitination also failed to catalyze ubiquitination of Cln1 (Figure 5D), despite the fact that Cln1 can associate, albeit weakly, with Cdc4 (Figure 5A). Identical preparations of phosphorylated Cln1 protein were efficiently ubiquitinated in partially purified yeast lysates in a Cdc34-dependent manner (Figure 5E and data not shown), indicating that this preparation of Cln1 is competent for ubiquitination. The absence of Cln1 ubiquitination in the purified system is therefore likely to reflect the requirement of additional factors or modifications.

Discussion

In this study, we have demonstrated that Sic1 is ubiquitinated in vivo and have reconstituted the pathway for Sic1 ubiquitination in vitro using recombinant proteins. This has allowed us to establish the following: (1) Skp1, Cdc53, and Cdc4 form a functional E3 ubiquitin ligase complex that works together with the E2 Cdc34 to ubiquitinate Sic1; (2) Cdc4 acts as a receptor for phosphorylated Sic1 recognition; and (3) the sole function Cln/Cdc28 kinases in this process is to phosphorylate Sic1, allowing recognition by Cdc4. Similar results are reported in the accompanying paper (Feldman et al., 1997 [this issue of *Cell*]). In addition, we have found that distinct F-box proteins can selectively recognize different ubiquitination substrates in a phosphorylation-dependent manner. We propose to call this Skp1/Cdc53/F-box protein complex SCF. Because interchangeable F-box proteins may serve as specificity determinants, we propose to define the complex by designating the F-box protein with a superscript, e.g., SCF^{Cdc4}.

A Cdc53/Cdc4/Skp1 E3 Complex Is Required for Sic1 Ubiquitination by Cdc34

Our studies demonstrate that Cdc34, Cdc4, Cdc53, and Skp1 are directly involved in Sic1 ubiquitination. While the precise roles of individual components of this complex are not fully understood, some general conclusions

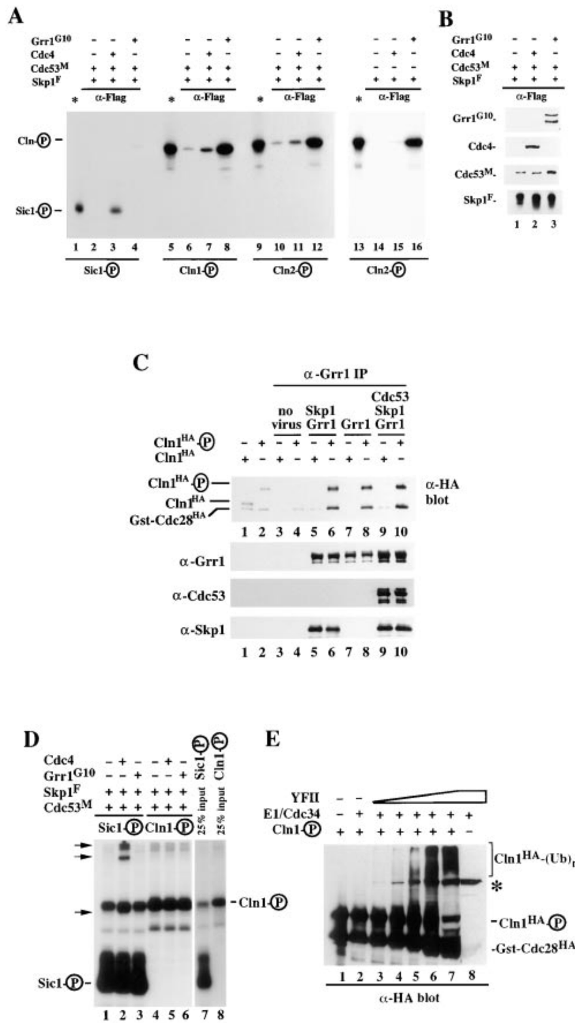

Figure 5. Selective Recognition of Phosphorylated Cln/Cdc28 Complexes by Grr1

(A) Differential recognition of Sic1 and Cln proteins by Grr1 and Cdc4. ^{32}P-labeled Sic1 or Cln complexes were incubated with the indicated anti-Skp1^{F} immune complexes, and bound proteins visualized by SDS–PAGE and autoradiography. Controls for the extent of binding (indicated by the asterisk) were 20% of input Cln and 10% of input Sic1.

(B) Complexes used for binding experiments in (A) were immunoblotted to verify the presence of Cdc4, Grr1^{G10}, Cdc53^{M}, and Skp1^{F}.

(C) Cln1 phosphorylation is required for its association with Grr1. Anti-Grr1^{G10} complexes (lanes 4–11) were used in binding reactions with either unphosphorylated Cln1^{HA} complexes generated using kinase-impaired Gst-Cdc28(K−)^{HA} (lane 1) or phosphorylated Cln1^{HA}/Gst-Cdc28^{HA} complexes (lane 2). Bound proteins were separated by SDS–PAGE and immunoblotted with anti-HA antibodies to detect Cln1^{HA} and Gst-Cdc28^{HA}. Twenty percent of the input Cln1^{HA} complexes were run as controls (lanes 1 and 2). Cln1^{HA} isolated from insect cells in complexes with active Cdc28 migrates as a series of modified forms reflecting partial phosphorylation of Cln1 (see Figure 2A). Incubation of Cln^{HA}/Cdc28^{HA} with ATP shifts Cln1^{HA} to a single form migrating ~84 kDa. The blot was reprobed to verify the presence of Grr1^{G10}, Cdc53^{M}, and Skp1^{F}.

(D) Skp1/Cdc53/Grr1 complexes are not sufficient for Cln1 ubiquitination by Cdc34 in vitro. Anti-Skp1^{F} immune complexes were purified from insect cells infected with the indicated viruses and ubiquitination reactions performed as in Figure 3C, except that ^{32}P-labeled Cln1 or Sic1 was used. After 60 min at 25°C, proteins were separated by SDS–PAGE and detected by autoradiography.

(E) Phosphorylated Cln1 is ubiquitinated in a fractionated yeast extract system. YFII (0–100 mg) was supplemented with Cdc34, human E1, ubiquitin, and an ATP-regenerating system, and the ubiquitination reaction initiated by Cln1^{HA} addition. After 60 min at 25°C, proteins were immunoblotted with anti-HA antibodies to detect Cln1^{HA} and Gst-Cdc28^{HA}. The protein in YFII indicated by an asterisk cross-reacts with anti-HA antibodies.

can be drawn. Cdc53 can simultaneously bind the E2 Cdc34 and Skp1, and functions as an adapter linking the recognition component (Skp1/F-box protein complex) to E2s (Figure 1). In turn, Skp1 has the ability to link Cdc4 to Cdc53. Cdc4 binds both Skp1 and the ubiquitination substrate Sic1. The interaction of Cdc4 with Skp1 involves the F-box located in the N terminus of Cdc4 (Bai et al., 1996), while the interaction with Sic1 involves Cdc4's C-terminal WD-40 repeats (Figure 2). Skp1 is involved in substrate recognition because it enhances the association of Cdc4 with phosphorylated Sic1. It remains to be determined whether substrates also contact Skp1 or whether Skp1 functions in recognition by stabilizing the appropriate conformation of Cdc4. In any event, Cdc4 acts as a receptor that together with Skp1 recruits substrates to the ubiquitination complex. In principle, any of these proteins could also have carrier roles in the transfer of ubiquitin like E6AP (Scheffner et al., 1995) However, we have found that mutation of the only conserved cysteine in Skp1 (P. Sen and S. J. E., unpublished data) or all 6 cysteines in Cdc53 (K. L. C. and M. T., unpublished data) did not impair complementation of *skp1* or *cdc53* null mutations, respectively, indicating that these two proteins are unlikely to transfer ubiquitin by a thio-ester intermediate.

F-Box Proteins as Receptors for Ubiquitination Targets

A large number of proteins contain the F-box and are thereby implicated in the ubiquitin pathway (Bai et al., 1996). Our analysis of Cdc4 and Grr1 has revealed that F-box proteins directly contact ubiquitination substrates and can display selectivity in recognition of potential targets for ubiquitination, as would be expected of E3 proteins. Although Cdc53 was isolated as a Cln2-interacting protein (Willems et al., 1996), it is probable that this original interaction was bridged by Grr1 and possibly Cdc4. The Grr1/Cln interaction is interesting in view of the fact that *GRR1*, *CDC53*, and *SKP1* are required for destruction of Cln proteins (Barral and Mann, 1995; Bai et al., 1996; Willems et al., 1996), and suggests that Grr1 functions as a component of an E3 for Cln ubiquitination. The absence of Cln ubiquitination by recombinant Grr1 complexes is likely to indicate the absence of an essential factor(s) or modifications that are not required for Sic1 ubiquitination in vitro, and suggests that Cln ubiquitination may be more complex than is Sic1 ubiquitination.

Individual F-box proteins are likely to recognize multiple substrates. In S. pombe, the *CDC4* homolog *pop1*$^{+}$ is required for ubiquitination of both the CKI Rum1 and Cdc18, a regulator of DNA replication (Kominami and Toda, 1997). In budding yeast, *CDC4* has also been implicated in destruction of the Cdc18 homolog Cdc6

(Piatti et al., 1996), indicating that it too has multiple targets. Of potential importance is the fact that all of the targets of F-box protein–mediated destruction identified to date are central regulators of key events in the cell, including DNA replication, cell cycle progression, and nutritional sensing.

Phosphorylation Directly Regulates Association of Sic1 and Cln Proteins with E3s

A central feature in the recognition of Sic1 and Cln by F-box proteins is the phosphorylation dependence. Cln2 phosphorylation is required for its instability and association with Cdc53 complexes in vivo (Lanker et al., 1996; Willems et al., 1996). While the identity of the kinase regulating Cln phosphorylation and E3 recognition in vivo is unknown, we have shown that autophosphorylation is sufficient to target Clns to Grr1 in vitro. If Cln ubiquitination is activated by autophosphorylation in *trans*, the accumulation of active Cln/Cdc28 complexes may be required to achieve sufficient Cln phosphorylation to promote its destruction. This logic provides a built-in delay between Cln activation and Cln destruction and allows time for other Cln substrates such as Sic1 to be phosphorylated prior to Cln destruction. Sic1 phosphorylation allows binding to Cdc4/Skp1 complexes; however, it is not clear at present whether phosphorylation is also required in the Sic1 ubiquitination step following binding. Although Cln/Cdc28 appears to play the primary role in vivo in initiating Sic1 ubiquitination, we have also observed that Clb5/Cdc28-phosphorylated Sic1 can be ubiquitinated (Figure 3E). The initial generation of Clb5/Cdc28 activity at the G1/S transition could potentially accelerate Sic1 destruction, facilitating the sharp and unidirectional change of state characteristic of cell cycle transitions.

While phosphorylation is an effective method controlling the timing of substrate recognition and ubiquitination, it is not necessarily the case that all F-box proteins will selectively recognize phosphorylated substrates. WD-40 and LRR containing F-box proteins can recognize phosphorylated substrates, but approximately half of the known F-box proteins do not have obvious protein interaction motifs (Bai et al., 1996). Whether these proteins will target phosphorylated substrates or even be involved in ubiquitination is unknown. In principle, it is possible that any protein that associates with the SCF could be a ubiquitination target and ubiquitination timing could be controlled by non-phosphorylation-dependent mechanisms such as controlled accessibility of substrates or regulated expression, localization, or modification of the F-box protein. While the abundance of Cdc4 is not cell cycle–regulated, the *SKP2* mRNA abundance is cell cycle–regulated and peaks in S phase, consistent with its association with cyclin A during that phase of the cycle (Zhang et al., 1995). In vivo, association of Grr1 and Skp1 is enhanced by glucose in a posttranslational mechanism (Li and Johnston, 1997). While this could be due to the presence of higher levels of Grr1-dependent substrates driving association with Skp1, it could also indicate a distinct regulatory step controlling Grr1 function and could explain our inability to observe Grr1-dependent Cln ubiquitination in vitro.

Is the Skp1 Pathway the PEST Sequence Proteolysis Pathway?

A large number of proteins contain PEST sequences, and in a subset of these, PEST sequences have been shown to be phosphorylated and to mediate instability. While the SCF^{Cdc4} complex we have defined is unlikely to be responsible for recognition of all PEST-dependent proteolysis substrates, it is likely to be the prototype for a diverse set of SCFs in higher eukaryotes whose targets will likely include many PEST-containing proteins. Five *CDC53* homologs have been identified in mammals (Cul1–5) (Kipreos et al., 1996), approximately 15 E2-related genes exist in S. cerevisiae alone, several dozen F-box containing proteins have been identified in several species (Bai et al., 1996 and data not shown), and multiple *SKP1*-related genes exist in C. elegans and are likely to exist in mammals as well. The number of complexes formed by combinatorial interactions of family members could provide the flexibility to differentially regulate the ubiquitination of a very large number of substrates.

Conserved Features of the SCF with Other Regulatory Pathways

Recent data indicate that the Skp1/F-box paradigm may have parallels in other settings. Elongin C, a Skp1-related protein (Bai et al., 1996), is part of a complex containing the Cdc53-related protein Cul2, the von Hippel-Lindau (VHL) tumor suppressor protein, and elongin B (Pause et al., 1997; W. Kaelin, personal communication). Elongin A, which is also found in association with elongin C (Aso et al., 1996), contains an F-box (Bai et al., 1996). Whether elongin C functions in a ubiquitination pathway or serves an alternative function is unclear at present. Interestingly, elongin B is related to ubiquitin and may replace ubiquitin in this pathway.

Some aspects of the SCF pathway may have parallels with another ubiquitination pathway, the APC. Recent data indicates that a Cdc53-related protein is part of the APC in Xenopus (H. Yu and M. Kirschner, personal communication) and in yeast (K. Nasmyth, personal communication). Given our observations, it is likely to be recruiting E2 proteins into the APC. In a further parallel, the WD-40-containing proteins Cdc20 and Cdh1/Hct1 appear to function in recognition of APC ubiquitination targets and therefore are likely to act as counterparts to Cdc4. (Schwab et al., 1997; Visintin et al., 1997). Presumably, there will be a function analogous to Skp1 that links the specificity factors to the Cdc53-related factors. That WD-40-repeat proteins are employed in both settings raises the possibility that the APC might also use protein phosphorylation in some capacity to regulate timing of substrate ubiquitination.

How the SCF Allows Protein Kinases to Control Protein Abundance

The SCF complexes described here provide one example of a pathway through which protein kinases control the stability of target proteins. Given the large number of protein kinases and possible SCFs, this pathway may be second only to transcriptional regulation in the control of protein abundance. While the examples described here concern the cell cycle, it is likely that this is a

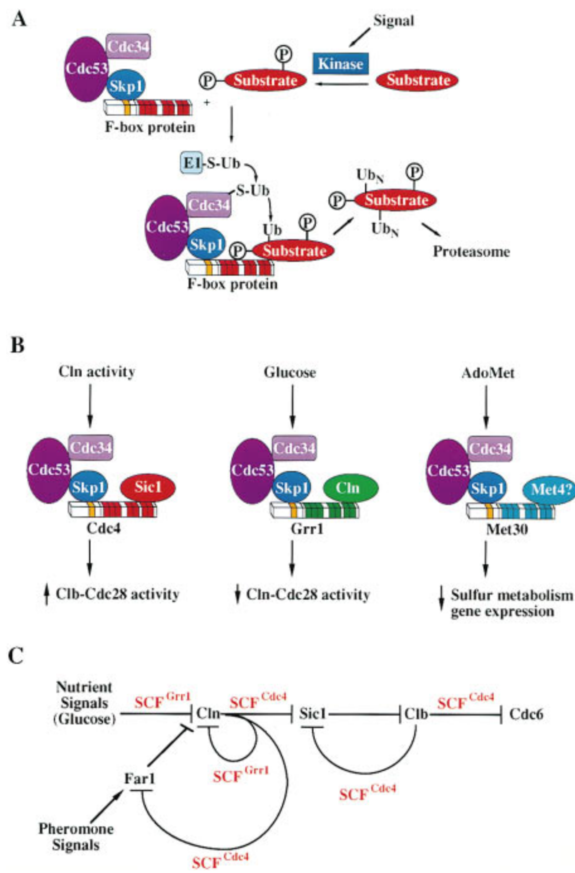

Figure 6. Models Depicting the Role of the SCF Complex in the Recognition and Ubiquitination of Targets

(A) Phosphorylation of substrates through protein kinase signaling pathways is required for recognition by F-box receptor proteins (see text for details).

(B) Distinct F-box complexes may regulate different biological processes through selective recruitment of substrates. SCF configurations are shown together with the signals that are being sensed, the corresponding substrates, and the physiological consequences of complex function. Not all components pictured in the SCF^{Met30} complex have been genetically linked to the process shown and remain hypothetical.

(C) The interplay between protein kinases and the SCF pathway in the G1 to S-phase transition in S. cerevisiae. Perpendiculars indicate inhibitory events.

general ubiquitination machine that can be used for diverse regulatory needs. We envision the model shown in Figure 6A in which a protein kinase phosphorylates target proteins, thus activating them for association with their F-box protein receptors. Although some F-box proteins may already be associated with a Skp1/Cdc53 complex prior to association with substrates, as shown in Figure 6A, it is also possible that F-box proteins exist in an unbound form, and that association of the F-box protein with the substrate drives association with Skp1/Cdc53. Since Skp1 enhances the association of Cdc4 with Sic1, depending on the relative K_d values for individual interactions and concentrations of the constituents, association of the target with an F-box protein may enhance association with Skp1. Once the ubiquitination complex is formed and polyubiquitination takes place with the assistance of E1 and E2 proteins, the substrate is then released and destroyed by the proteasome. Other combinations of SCF complexes are likely to exist in the cell (Figure 6B). For example, the F-box protein Met30 is closely related Cdc4 and is required for repression of genes in the methionine biosynthetic pathway in the presence of S-adenosylmethionine (AdoMet) (Thomas et al., 1995). Met30 forms complexes with Met4, a transcription factor required for methionine biosynthetic gene expression, and also associates with Skp1 and Cdc53 (E. Patton and M. T., unpublished data). Our model would predict that Met4 is ubiquitinated in response to adomethionine. Although our studies have focused on Cdc34, other E2s may also be capable of functioning in the context of SCFs. Furthermore, these SCF complexes work hand in hand with protein kinase signaling pathways to control protein abundance. An example of the tightly interwoven nature of these pathways is illustrated in Figure 6C, which illustrates how SCF pathways function multiple times in the transition from G1 to S phase in S. cerevisiae.

Like protein synthesis, protein destruction is a fundamental mechanism organisms employ to manipulate their function. We have elucidated the composition of an E3 complex involved in selection of ubiquitination substrates. Because the constituents of this complex are members of protein families, it is likely to serve as the prototype for a large class of E3s formed by combinatorial interactions of related family members as indicated in Figure 6B. The identification of F-box proteins as the receptor components of this ubiquitin ligase should facilitate the identification of the key regulatory molecules controlled by ubiquitin-mediated proteolysis. The elucidation of the biochemistry of this general ubiquitination pathway is likely to have important ramifications for many aspects of biology including cell proliferation, development, and differentiation.

Experimental Procedures

Antibodies

Anti-Skp1 and anti-Sic1 were generated in rabbits using bacterial Gst fusion proteins as antigen. Polyclonal rabbit anti-Cdc34 and anti-Cdc4 sera (provided by M. Goebl) as well as anti-Sic1 were purified using nitrocellulose-bound antigens. Monoclonal antibodies: anti-HA and anti-Myc (Babco), anti-T7 gene10 (referred to as G10, Novagen), and anti-Flag (Kodak). Antibody detection was by ECL (Amersham).

Purification and Phosphorylation of Recombinant Proteins

Baculoviruses (Table 1) were generated using linearized BaculoGold or AcMNPV wild-type DNA (Pharmingen). Cdc4ΔWD is a mutant version of Cdc4 that contains a stop codon at residue 566 that removes the last three WD-40 repeats. Gst-$Cdc28^{HA}$ (D154N), referred to as Gst-$Cdc28^{HA}$(K−), is a kinase-impaired form of Cdc28. His_6-Cdc34 and His_6-Sic1 (in pET vectors, Novagen) were expressed in E. coli (BL21 DE3) and purified on Ni^{+2}-NTA (Qiagen) resin as suggested by the manufacturer.

For protein expression, insect cells (Hi5, Invitrogen) were infected with the indicated virus combinations for 40 hr. Cells were disrupted in lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.5% Nonidet P-40, 10 mM NaF, 10 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, antipain, and aprotinin) and cleared by centrifugation. Typically, 3 ml of lysis buffer was used per 0.5×10^8 cells. For affinity or immunopurification, 0.3–0.6 ml of cell lysate was incubated at 4°C for 2 hr with 4 μg of the anti-Myc or anti-G10 antibody and 8 μl of protein A–Sepharose, or with

Table 1. Baculoviruses

Virus	Tag	Base Vector	Source
Cak1	None	pVL	This study[a]
Cdc4	None	pBBIII	This study
Cdc4ΔWD	None	pBBIII	This study
$Cdc4^F$	C-terminal Flag	pBBIII	This study
Cdc34	None	pBBIII	This study
$Cdc53^M$	N-terminal Myc	pBBIII	Willems et al. (1996)
Clb5	None	pVL	B. Stillman
$Cln1^{HA}$	C-terminal HA	pBBIII	This study
$Cln2^{HA}$	C-terminal HA	pVL	This study
Gst-$Cdc28^{HA}$	N-terminal Gst, C-terminal HA	pVL	This study
Gst-$Cdc28^{HA}$ (D145N)	N-terminal Gst, C-terminal HA	pVL	This study
$Grr1^{G10}$	N-terminal His_6-G10	pBBHis	This study[b]
His_6-Cks1	N-terminal His_6	pVL	This study
Sic1	None	pBBIII	This study
Skp1	None	pVL	This study
$Skp1^F$	N-terminal Flag	pBBIII	This study
Gst-Skp1	N-terminal Gst	pVL	This study

[a] *CAK1* gene provided by C. Mann.
[b] *GRR1* gene provided by M. Johnston.

8 μl of immobilized anti-Flag antibodies. Immune complexes were washed three times with 1 ml of lysis buffer prior to SDS–PAGE and immunoblotting.

Sic1/Clb5/Gst-$Cdc28^{HA}$(K−) was purified from 4 × 10^8 cells using 200 μl of GSH-Sepharose and eluted with 40 mM glutathione in 100 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5% glycerol. Gst-$Cdc28^{HA}$/Cln^{HA}/Cks1 and kinase-impaired Gst-$Cdc28^{HA}$(K−)/$Cln1^{HA}$/Cks1 complexes were prepared similarly from cells coinfected with viruses expressing appropriate proteins and CAK1 expressing virus. The presence of Cks1 and Cak1 resulted in an increase of the yield of active Cln/Cdc28 kinase complexes.

For Sic1 phosphorylation, Sic1/Clb5/Gst-$Cdc28^{HA}$(K−) (2.5 μM) was incubated with Gst-$Cdc28^{HA}$/$Cln1^{HA}$/Cks1 (50 nM) and 1 mM ATP in 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$ for 45 min at 25°C. Unphosphorylated Sic1 complexes were produced by omitting Cln1 kinase. Cln/Cdc28 autophosphorylation was performed by incubating 200 nM Cln/Cdc28 complexes with 1 mM ATP in kinase buffer at 25°C for 1 hour. To generate phosphorylated Sic1 free of Cln/Cdc28 kinase, bacterial Sic1 (0.5 μM) was incubated with 2 mM ATP and Cln2/Gst-Cdc28/Cks1 immobilized on GSH-Sepharose (60 min at 37°C). Phosphorylated Sic1 (40 ng) was removed from the beads for use in ubiquitination reactions, at a final concentration of 1 nM. For ^{32}P-labeling of Sic1 and Cln proteins, kinase reactions were performed at 25°C for 30 min using 50 μM [γ-^{32}P] ATP (0.3 nCi/pmol) followed by incubation with 1 mM ATP for 30 min.

In Vitro Binding Assays

Binding reactions were performed at 4°C for 1 hour in 100–250 μl mixtures containing appropriate immunopurified complexes and affinity-purified Sic1 (20 nM) or Cln (2 nM) complexes. Associated proteins were then washed three times with 1 ml of lysis buffer prior to SDS–PAGE and immunoblotting. In some experiments, ^{32}P-labeled Sic1 or Cln complexes were employed and detected by autoradiography and phosphoimager analysis. Based on protein staining with Coomassie Blue or silver, we estimate the quantities of proteins in anti-$Skp1^F$ immune complex from $Skp1^F$/$Cdc53^M$/Cdc4 expression cells to be: $Skp1^F$ (1 μg), $Cdc53^M$ (200 ng), and Cdc4 (200 ng). Likewise, the levels of proteins in the anti-$Grr1^{G10}$ complex were: $Grr1^{G10}$ (100 ng), $Cdc53^M$ (40 ng), and Skp1 (20 ng).

Ubiquitination Assays

Ubiquitination reactions contained immune complexes prepared from 2 × 10^6 cells and equilibrated with ubiquitination buffer (100 mM Tris–HCl [pH 7.5], 5 mM $MgCl_2$, 0.6 mM DTT), 500 nM bacterial Cdc34, 300 nM human E1 (from M. Rolfe, Mitotix), 2 mM ATP, and 7 μM yeast ubiquitin (Sigma) or Gst-Ub^{RA} (purified from bacteria) and 80 ng of Sic1 complexes in a volume of 14 μl excluding beads. Reactions were allowed to proceed at 25°C for 1 hour or as indicated prior to SDS-PAGE and immunoblotting with anti-Sic1 antibodies. In some experiments, bacterial Sic1 was used and where indicated, was phosphorylated with soluble or immobilized Gst-Cdc28/Cln2. Bacterial Sic1 ubiquitination reactions employed 100 nM yeast E1 (a gift from S. Sadis and D. Finley). Crude yeast extracts (Deshaies et al., 1995) were chromatographed on DEAE-Sephacryl. Proteins eluting with 250 mM KCl (YFII) were used for ubiquitination. Ubiquitination reactions (20 μl) contained the indicated quantity of YFII, 500 nM Cdc34, 300 nM human E1, 2 mM ATP, an ATP regeneration system, 20 μM ubiquitin, 20 ng of autophosphorylated Cln1/Gst-Cdc28/Cks1. Ubiquitination of Cln1 by YFII required addition of Cdc34 and ubiquitin (data not shown).

To identify Sic1-ubiquitin conjugates in vivo, 200 ml (10^7 cells/ml) of wild-type (MT235) or a *sic1* deletion (MT767) cells expressing either *pCUP1-UBI*^{RA} (<pUB204>) or *pCUP1-UBI*$^{HIS\text{-}MYC\text{-}RA}$ (<pUB223>) were prepared and lysates generated as described (Willems et al., 1996). Purification of conjugates from 8 mg of yeast proteins was achieved using 12 μl of Ni^{+2}-NTA beads (1 hr at 4°C). Beads were washed three times in lysis buffer, once with 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and proteins eluted prior to SDS–PAGE and immunoblotted with anti-Sic1 antibodies.

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