

[13] *In Vitro* Reconstitution of SCF Substrate Ubiquitination with Purified Proteins

By MATTHEW D. PETROSKI and RAYMOND J. DESHAIES

Abstract

The development of *in vitro* systems to monitor ubiquitin ligase activity with highly purified proteins has allowed for new insights into the mechanisms of protein ubiquitination to be uncovered. This chapter describes the methodologies employed to reconstitute ubiquitination of the budding yeast cyclin-dependent kinase inhibitor Sic1 by the evolutionarily conserved ubiquitin ligase SCF^{Cdc4} and its ubiquitin-conjugating enzyme Cdc34. Based on our experience in reconstituting Sic1 ubiquitination, we suggest some parameters to consider that should be generally applicable to the study of different SCF complexes and other ubiquitin ligases.

Introduction

The development of *in vitro* systems with highly purified proteins allows for the molecular mechanisms of the steps involved in the ubiquitination of a protein to be directly explored and dissected. We have developed such a system to study the ubiquitination of the budding yeast cyclin-dependent kinase (CDK) inhibitor Sic1 by the ubiquitin ligase SCF^{Cdc4} and the ubiquitin-conjugating enzyme Cdc34. Whereas genetic studies initially identified some of the proteins involved in this process, it was not until biochemical approaches were developed and applied that all of the proteins necessary and sufficient for Sic1 ubiquitination were identified (Feldman *et al.*, 1997; Kamura *et al.*, 1999; Seol *et al.*, 1999; Skowrya *et al.*, 1997, 1999; Verma *et al.*, 1997b).

Sic1 binds to and inhibits the activity of the S-phase cyclin-dependent kinase complex (S-CDK) during the G1 phase of the budding yeast cell cycle (Mendenhall, 1993; Schwob *et al.*, 1994). As the level of G1 phase CDK (G1-CDK) activity increases, Sic1 becomes multiply phosphorylated at consensus CDK sites (Nash *et al.*, 2001; Verma *et al.*, 1997a). Phosphorylated Sic1 is recognized by SCF^{Cdc4} and becomes ubiquitinated through the activity of Cdc34 (Verma *et al.*, 1997b). Once ubiquitinated, Sic1 is degraded rapidly by the 26S proteasome, resulting in activation of S-CDK and allowing for progression into the S phase of the cell cycle (Verma *et al.*, 2001).

Sic1 destruction at the G1–S phase boundary represents an important model system for understanding the mechanisms of protein ubiquitination and degradation. *In vitro* reconstitution of this process has been used successfully to study how SCF recognizes phosphorylated substrates (Nash *et al.*, 2001; Orlicky *et al.*, 2003; Verma *et al.*, 1997a), the mechanisms of ubiquitin transfer through SCF (Deffenbaugh *et al.*, 2003), the minimum set of components that can sustain Sic1 turnover and S-CDK activation (Verma *et al.*, 2001), the number and position of ubiquitin chains that can promote 26S proteasome-mediated degradation (Petroski and Deshaies, 2003), and the role of targeting factors in guiding ubiquitinated Sic1 to the 26S proteasome (Verma *et al.*, 2004). As a result, SCF is perhaps the best understood member of the RING class of ubiquitin ligases. These ligases, in contrast with HECT domain-containing ligases, do not form a catalytic intermediate with ubiquitin, but instead may activate through the RING motif the direct transfer of ubiquitin from the active site cysteine of the ubiquitin-conjugating enzyme onto the lysine of the ubiquitin ligase-bound substrate. RING proteins are probably the most common type of ubiquitin ligase, but the molecular basis of how they work remains largely unknown.

SCF consists of Skp1, Cul1, a variable F-box protein that confers substrate specificity, and the RING-H2 subunit known alternatively as Hrt1, Rbx1, or Roc1 (for a review, see Petroski and Deshaies, 2005). SCF has been implicated in many aspects of cellular and organismal homeostasis, including cell cycle control, transcription, development, and circadian clock regulation. Cul1 is a member of the cullin family of proteins, of which at least five of the mammalian family members have been shown to form similar ubiquitin ligases with an enzymatic core that is targeted to different substrates by variable substrate recognition components. It seems highly likely, therefore, that paradigms established by studying how ubiquitin transfer through SCF works will apply not only to other cullin-based ubiquitin ligases, but also to RING-based ligases in general.

This chapter describes the strategies and methods developed to study the ubiquitination of Sic1 by SCF^{Cdc4}. It also discusses efforts to develop substrates containing defined ubiquitin-accepting sites, which are useful for studies aimed at unraveling the mechanism of ubiquitination (see Petroski and Deshaies, 2003). We describe the methods we have developed for the expression and purification of proteins involved in this process and the reaction conditions we use to reconstitute the ubiquitination of Sic1 by SCF^{Cdc4} *in vitro*. We expect that these approaches will be adaptable and applicable to the study of substrates of other ubiquitin ligases.

General Considerations for the Development of Ubiquitin Ligase Substrates

When developing an *in vitro* assay to examine the ubiquitination of a particular protein by its ubiquitin ligase, it is important to consider several parameters likely to have a significant impact on the probability of success. Many ubiquitin ligase substrates (particularly those of cullin-based ligases) require some sort of posttranslational modification, such as phosphorylation for recognition. This will influence the choice of expression system used to generate sufficient quantities of the substrate of interest. Perhaps the easiest and most robust approach to generate large quantities of protein is by bacterial expression. However, this system has the drawback of lacking eukaryotic posttranslational modifications. While baculovirus expression systems may require slightly more expertise and considerably more time, posttranslational modifications may occur during expression in insect cells, potentially eliminating the requirement for pretreating substrates prior to use in ubiquitination assays. This may be particularly advantageous if the proteins involved in the modification are unknown. However, if degradation-promoting modifications occur in insect cells, the substrate may be degraded if its cognate ubiquitin ligase is present. Thus, in some circumstances, it may be necessary to apply chemical inhibitors of the proteasome several hours prior to harvesting cells. Other eukaryotic systems such as yeast or *in vitro* transcription/translation with reticulocyte lysates (e.g., see [Verma *et al.*, 1997a](#)) are possible alternatives to insect cells.

In attempting to elucidate sites of ubiquitin attachment on a substrate, it is important to note that some substrates may be ubiquitinated at the N terminus instead of exclusively at lysine residues (see, for example, [Aviel *et al.*, 2000](#); [Bloom *et al.*, 2003](#); [Fajerman *et al.*, 2004](#); [Ikeda *et al.*, 2002](#); [Reinstein *et al.*, 2000](#)). In our efforts to identify the sites of Sic1 ubiquitination, we mutated all 20 lysine residues of Sic1 to arginine ([Petroski and Deshaies, 2003](#)). This lysine-less version of Sic1 is stable *in vivo* and cannot be ubiquitinated *in vitro* by SCF^{Cdc4}, suggesting that N-terminal ubiquitination of Sic1 does not occur. During the course of these studies, we also determined that it is important to ensure that fusion proteins or epitope tags used to facilitate purification and/or detection do not contain lysines, as these introduced residues may become sites of ubiquitin attachment which can, in turn, complicate analyses.

Traditionally, ubiquitination sites are mapped by mutating lysine residues to arginine. Unfortunately, several of the tRNAs for arginine are exceptionally rare in *Escherichia coli* (see [Table I](#)), which may have the effect of reducing expression of the mutated protein or result in an

TABLE I
FREQUENCY OF CODON USAGE FOR LYSINE AND ARGININE tRNAs IN *E. coli*^a

Amino acid	Codon	Frequency ^b
Lysine	AAG	0.24
	AAA	0.76
Arginine	AGG	0.02
	AGA	0.04
	CGA	0.06
	CGG	0.10
	CGU	0.38
	CGC	0.40

^a Arginine codons that are utilized infrequently (less than 10% of total usage) are indicated in bold. It is advisable when mutating lysine residues to arginine to introduce CGG or CGT instead of sequences corresponding to the four rare arginine codons or to use bacterial strains that may correct for low-level expression of these tRNAs.

^b From Nakamura *et al.* (2000).

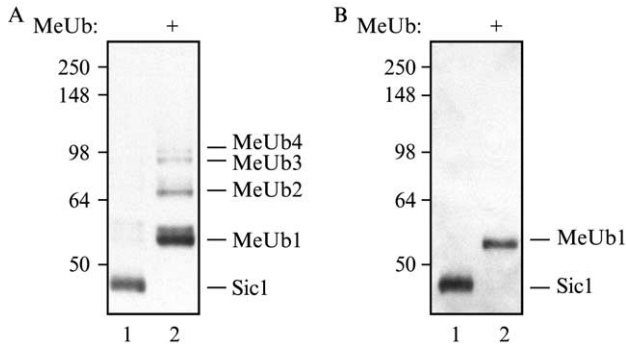


FIG. 1. Influence of rare codon usage on the ubiquitination pattern of *E. coli* expressed Sic1. (A) Sic1 containing a single lysine residue (K32) was expressed and purified from the BL21(DE3)pLyS *E. coli* strain (Novagen), phosphorylated, and utilized in an *in vitro* ubiquitination reaction containing Uba1, Cdc34, SCF^{Cdc4}, methyl ubiquitin, and ATP. Lane 1 is the reaction performed without added MeUb and lane 2 is the complete reaction. The number of methyl ubiquitin (MeUb) attachments onto Sic1 is indicated. As lysine residues are required for Sic1 destabilization *in vivo* and this substrate contains only a single lysine (K32) that confers destabilization *in vivo* (Petroski and Deshaies, 2003), secondary sites of MeUb attachment presumably arise from misincorporation of extra (i.e., not genetically encoded) lysines into Sic1 during *E. coli* expression. (B) Sic1 containing a single lysine residue was expressed and purified from BL21-Codon Plus *E. coli* strain (Stratagene) and utilized in an *in vitro* ubiquitination reaction as in A. Note that only a single MeUb was incorporated into this substrate, suggesting that expression of the tRNAs for rare arginine codons in this strain circumvented the misincorporation problem shown in A.

unacceptably high rate of misincorporation of amino acids when using conventional bacterial expression systems (You *et al.*, 1999). The latter problem is highlighted by analysis of a Sic1 substrate containing a single lysine residue that was expressed in BL21(DE3)pLysS and used in ubiquitination assays employing methyl ubiquitin (Fig. 1A). Surprisingly, multiple ubiquitinated species were detected, which indicates the presence of more than one ubiquitin attachment site. Possible solutions to this problem include minimizing protein induction times or utilizing *E. coli* strains (e.g., the Rosetta strains from Novagen or BL21-Codon Plus strains from Stratagene) that compensate for these rare tRNAs by harboring extra copies of these genes. As shown in Fig. 1B, expression of single lysine Sic1 in BL21-Codon Plus-RIL eliminated the problem illustrated in Fig. 1A.

It is also worth noting that preexisting knowledge might reduce the amount of effort required to identify potential targets for ubiquitin conjugation on a substrate. For example, Sic1 is bound to the S-CDK protein complex prior to its degradation (Nugroho and Mendenhall, 1994). While “naked” Sic1 can be ubiquitinated on a large fraction of its 20 lysine residues, this number is reduced significantly when Sic1 is assembled into its “physiological” context prior to its ubiquitination *in vitro* (see Petroski and Deshaies, 2003) (Fig. 2).

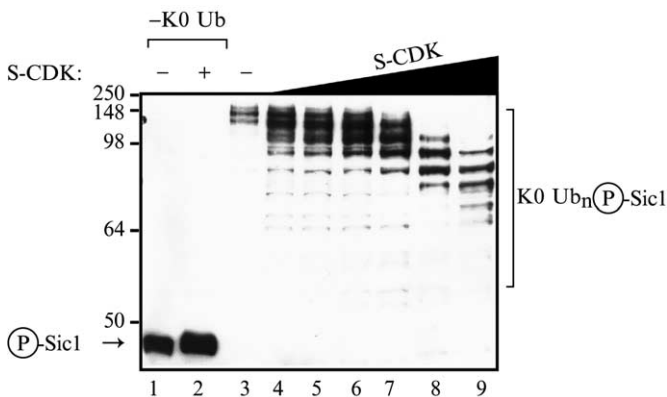


FIG. 2. Suppression of sites of Sic1 ubiquitination by the presence of S-CDK. Sic1 was preincubated in the absence ($-S\text{-CDK}$) or presence ($+S\text{-CDK}$) of purified S-CDK prior to addition to ubiquitination reactions containing Uba1, Cdc34, SCF^{Cdc4} , K0 ubiquitin, and ATP. The relative position of Sic1 and the position of K0 ubiquitin (K0 Ub) attachments onto Sic1 are indicated. Note that Sic1 in the presence of saturating amounts of S-CDK is ubiquitinated on up to six or seven sites whereas considerably more sites are utilized in the absence of S-CDK. Reprinted from Petroski and Deshaies (2003), with permission.

Site-Directed Mutagenesis of the Sic1 Open Reading Frame

Standard cloning procedures are used to generate a bacterial expression construct containing the Sic1 open reading frame in pET11b (Novagen). Sequences for an N-terminal T7 epitope tag (MASMTGGQQMG, Novagen) and a C-terminal hexa-histidine were also included. To generate various lysine to arginine mutations, this plasmid is subjected to site-directed mutagenesis following the Quikchange protocol (Stratagene). In general, oligonucleotides are designed such that the desired mutation is centered on the two complementary oligonucleotides with approximately 15 nucleotides on both sides. Typical polymerase chain reaction (PCR) conditions are used with a 68° extension for 2 min per kilobase of plasmid and a total of 15 cycles.

To ensure high mutagenesis efficiency, it is important to optimize the amount of input plasmid to use as little as possible. It is also possible to introduce multiple mutations simultaneously, either by introducing them on the same oligonucleotides if the sites are sufficiently close together or by using multiple oligonucleotides in the same reaction (Quikchange multisite-directed mutagenesis, Stratagene). Introduction of the desired mutation is confirmed by DNA sequencing.

Expression and Purification of Proteins

Uba1 Expression and Purification from Yeast

Both yeast and human E1 are available for purchase from Boston Biochem and Affiniti. Alternatively, baculoviruses for insect cell expression or purification from rabbit reticulocyte lysates have been utilized as E1 sources.

1. Grow an overnight culture of yeast strain RJD941 in YPD. This strain contains *Saccharomyces cerevisiae* *UBA1* encoding a hexa-histidine C-terminal tag under control of the copper-inducible *CUPI* promoter.
2. Inoculate 1 liter of YPD with 4 ml overnight culture.
3. Grow to saturation (at 36 h at 30°).
4. Add copper sulfate to a final concentration of 1 mM.
5. Grow an additional 24 h.
6. Pellet cells and wash once with water.
7. Resuspend in 25 ml buffer A (20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 10 mM imidazole, 5 mM MgCl₂, 10% glycerol).
8. Lyse cells in Constant Systems One Shot at 25 kpsi (mechanical grinding under liquid nitrogen in a mortar and pestle also works).

9. Pellet insoluble material at 50,000g for 30 min.
10. Add supernatant to 3 ml NiNTA agarose (Qiagen) and incubate at 4° for 1 h.
11. Wash four times with buffer A + 0.25% Triton X-100 (usually done in batch format, although column chromatography works as well).
12. Elute three times with 10 ml buffer B (20 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.5 M imidazole, 5 mM MgCl₂). Each elution is done in batch for 3 min at room temperature. Usually, the majority of E1 comes off in the first elution.
13. Dialyze eluate against 30 mM Tris-HCl, pH 7.6, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 15% glycerol (1 liter buffer with two changes, first one usually overnight).
14. Concentrate to desired concentration (greater than 1 mg/ml) in a Biomax (Millipore) centrifugal filter device. (If higher purity is desired, further purification steps such as ubiquitin affinity, gel filtration, or ion-exchange chromatography may be used. However, this single-step procedure generally results in >90% purity.) Typically, 2 mg of E1 is recovered per liter of culture from the NiNTA purification. Store at -80° in small aliquots. A Uba1 preparation is shown in Fig. 3A.

Cdc34 Expression and Purification from Bacteria

This protocol is used routinely to express and purify hexa-histidine-tagged proteins from *E. coli*.

The *S. cerevisiae CDC34* open reading frame appended to sequences encoding a C-terminal hexa-histidine tag was cloned by standard techniques into pET11b, transformed into BL21(DE3)pLysS (Novagen), and transformants were selected on LB plates containing ampicillin (50 µg/ml) and chloramphenicol (25 µg/ml).

1. Inoculate a 5-ml culture of LB medium containing ampicillin (50 µg/ml) and chloramphenicol (25 µg/ml) with a single colony from a fresh transformation and grow overnight at 37° with 200 rpm shaking.
2. Inoculate 1 liter of 2 XYT containing ampicillin (50 µg/ml) and chloramphenicol (35 µg/ml) with 5 ml of the overnight culture.
3. Grow to OD₆₀₀ of 1 at 30° with 200 rpm shaking and induce with 0.8 mM isopropyl-β-D-thiogalactoside for 2.5 h.
4. Pellet cells and wash once with 25 mM Tris-HCl, pH 7.6.

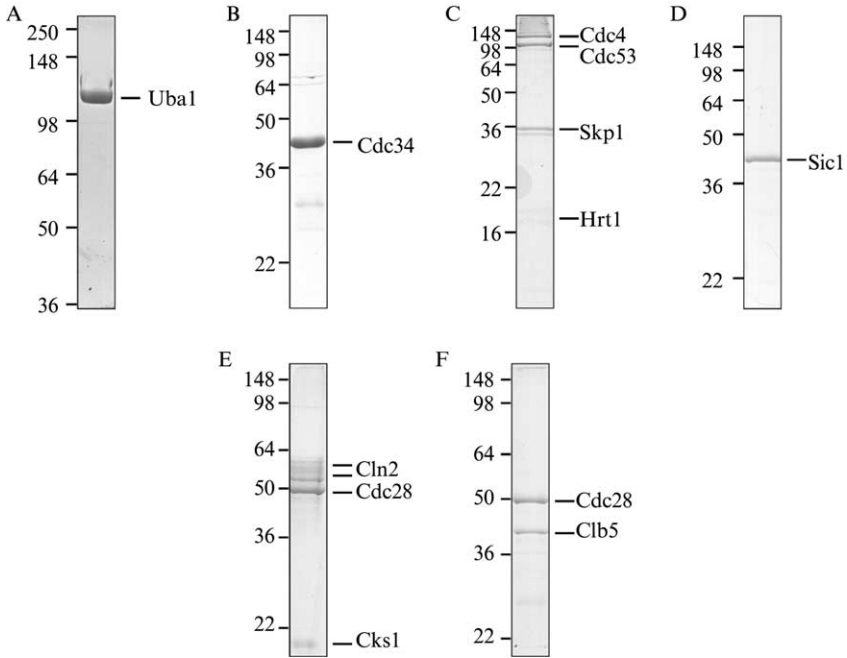


FIG. 3. Purified reaction components for Sic1 ubiquitination assays. (A) Uba1 was purified from yeast strain RJD941 by NiNTA chromatography. This strain expresses hexahistidine-tagged Uba1 under control of a copper-inducible promoter. A total of 2 μg was loaded and run on an 8% SDS-containing polyacrylamide gel followed by Coomassie staining. (B) Cdc34 was purified from *E. coli* strain BL21(DE3)pLysS transformed with a pET11b plasmid that contains the *CDC34* open reading frame fused to sequences that encode a C-terminal hexahistidine tag. A Coomassie-stained 12% polyacrylamide gel containing SDS loaded with approximately 2 μg is shown. (C) SCF^{Cdc4} was expressed in Hi5 insect cells by coinfection of baculoviruses encoding Py3HA2-tagged Cdc4, Cdc53, HA-tagged Skp1, and Hrt1. Forty-eight hours postinfection, lysates were prepared and SCF^{Cdc4} was purified on protein A-Sepharose beads that had been coupled with anti-Py antibodies. Approximately 1 μg of total protein was loaded onto a 15% gel and analyzed by Coomassie staining. (D) After expression in BL21-Codon Plus *E. coli*, a mutant of Sic1 that contains a single lysine (K32) was purified on NiNTA agarose. Approximately 1 μg from a typical purification that was analyzed on a 12% SDS-polyacrylamide gel by Coomassie staining is shown. (E) G1-CDK was prepared from Hi5 insect cells that were coinfecting with baculoviruses encoding GST-Cdc28HA, Myc-tagged Cln2, His6-tagged Cks1, and Cak1. The complex was purified on glutathione-Sepharose from lysates prepared after 48 h of infection. The resulting material (approximately 1 μg of total protein) was analyzed by SDS-PAGE on a 12% gel followed by Coomassie staining. (F) S-CDK was purified by glutathione-Sepharose chromatography from Hi5 insect cells that were coinfecting with baculoviruses encoding GST-Cdc28HA and Clb5. A Coomassie-stained 12% polyacrylamide gel loaded with approximately 1 μg of total protein is shown.

5. Resuspend pellet in 20 ml of 25 mM Tris-HCl, pH 7.6, and add imidazole to 10 mM, NaCl to 0.5 M, and Triton X-100 to 0.2%.
6. Freeze in liquid nitrogen.
7. Thaw to lyse cells (expression of T7 lysozyme from pLysS leads to cell lysis upon freeze-thaw) and sonicate briefly to reduce lysate viscosity.
8. Clarify lysate by centrifugation.
9. Add to 1 ml NiNTA agarose (Qiagen) and incubate at 4° for 30 min. (All purification steps are done in batch.)
10. Wash resin four times with 25 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 0.2% Triton X-100, 10 mM imidazole, and 10% glycerol.
11. Wash resin four times with 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl. (An ATP wash step for 5 min at room temperature with mixing of 20 mM Tris-HCl, pH 7.6, 2 mM ATP, and 5 mM MgCl₂ may reduce the amount of contaminating heat shock proteins, if desired.)
12. Elute three times for 2 min each with 3 ml 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 0.5 M imidazole.
13. Check eluate for protein by SDS-PAGE and pool fractions.
14. Dialyze against 30 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 10% glycerol (1 liter buffer, with two changes).

From a 1-liter culture, at least 50 mg of Cdc34 is recovered. A typical Cdc34 preparation is shown in [Fig. 3B](#).

SCF Expression and Purification from Baculovirus-Infected Insect Cells

To generate SCF^{Cdc4} in sufficient quantities for ubiquitination assays, we employ the baculovirus/insect cell expression system, as it allows for multiple proteins to be expressed simultaneously and relative expression levels of SCF subunits can be manipulated by altering the amount of the various viruses added to insect cells.

1. Baculoviruses containing the various open reading frames of *S. cerevisiae* SCF^{Cdc4} components (Skp1, Cdc53, Hrt1, and Cdc4) are prepared according to the manufacturer's instructions (Invitrogen). For ease of purification, *CDC4* contains sequences encoding an N-terminal tag comprising two repeats of the polyoma (Py, EYMPME) epitope followed by three repeats of the hemagglutinin (HA, YPYDVPDYA) tag. Alternative epitope tags may be used. The baculovirus encoding Skp1 also contains a C-terminal HA tag, although this is not used for purification.

2. Generate high-titer stocks of the individual viruses by infecting 50 ml of Sf9 insect cells (*Spodoptera frugiperda*, 1×10^6 cells/ml, 200-ml

spinner flasks) with 0.5 ml low titer stock. Grow for 3 to 4 days until cells are approximately 80% lysed as judged by trypan blue exclusion.

3. Seed Hi5 insect cells (Invitrogen) onto 10-cm² plates at 1×10^7 and allow cells to adhere.

4. Add high-titer virus stocks in appropriate ratios. (For SCF^{Cdc4}, 0.6 ml Py2HA3-Cdc4, 0.6 ml Cdc53, 0.3 ml Skp1-HA, and 0.3 ml Hrt1 are typically used. This was determined empirically to give the best yield of intact SCF^{Cdc4}.)

5. Incubate infected cells for 48 h at 27°.

6. Remove cells from plates and wash with phosphate-buffered saline (PBS).

7. Resuspend cell pellets in cold Sf9 lysis buffer (25 mM HEPES, pH 7.4, 250 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 1 mM DTT, 5 mM MgCl₂, 10% glycerol) using 2 ml buffer per 10-cm² plate.

8. Sonicate briefly and pellet cellular debris. At this point, the clarified lysates can be stored indefinitely at -80° or used directly to purify SCF^{Cdc4}.

9. Add lysates to protein A beads coupled to the anti-Py monoclonal antibody. Typically, 1 ml of anti-Py affinity resin is incubated with 10 ml lysate. Covalently coupled antibody-protein A beads are prepared as described (Harlow and Lane, 1988).

10. Incubate 3 h at 4°.

11. Wash three times with Sf9 lysis buffer. Wash four times with Py wash buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% Igepal CA-630, 1 mM EDTA, 1 mM DTT, 10% glycerol).

12. Elute 2 h at room temperature with 3 ml 100 µg/ml Py peptide (EYMPME) in Py wash buffer containing 300 mM NaCl, 0.1% *n*-octylglucoside, and 20 µg/ml arg-insulin. Elution efficiencies are typically 50 to 70%. A second elution may help increase yield. Beads can be reused numerous times (at least three) after stripping with 0.1 M glycine, pH 2, and reequilibration with Sf9 lysis buffer. (If soluble SCF is not required or desired, bead-bound SCF can be utilized. In this instance, the protocol can be terminated prior to washing with Py wash buffer and the beads can be equilibrated in ubiquitination reaction buffer prior to use.)

13. Concentrate SCF in a Biomax (Millipore) centrifugal filtration device. As 100 nM SCF is typically used in a 20-µl ubiquitination reaction, 2 µM is a desired final concentration. While it is difficult to get an exact concentration of fully assembled SCF due to variations in the stoichiometry of the possible complexes that may form, reasonable approximations can be made by performing a protein concentration assay and by Western blotting of gels containing titrations of the purified SCF to various known concentrations of purified subunits (e.g., individual subunits expressed and purified in *E. coli*). It is reasonable to expect 250 ng to 1 µg of SCF per 10-cm² plate by this method.

14. Dialyze eluted material against 30 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM MgCl₂, 2 mM DTT, and 15% glycerol after concentrating to desired volume and store at -80° in small aliquots.

A typical preparation of eluted SCF^{Cdc4} is shown in Fig. 3C.

Sic1 Expression and Purification from Bacteria

1. Transform a pET11b plasmid that encodes hexahistidine-tagged Sic1 into the bacterial strain BL21-Codon Plus-RIL (Stratagene) and plate cells onto LB medium with ampicillin (50 µg/ml) and chloramphenicol (25 µg/ml). Alternative strains that also contain genes that encode the rare tRNAs in *E. coli* such as Rosetta (Novagen) can also be used. This is important due to the high number of lysine→arginine replacements introduced into Sic1 mutants that contain a single lysine.

2. For expression and purification, follow the protocol described earlier for Cdc34.

3. Instead of dialysis, purify the eluted protein from NiNTA agarose by gel filtration on a PD10 column equilibrated with kinase buffer (20 mM HEPES, pH 8, 200 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT). Sic1 proteins containing a large number of lysine→arginine mutations tend to precipitate during dialysis. It was determined empirically that using gel filtration for buffer exchange circumvents this problem.

Sic1 purified by this protocol is shown in Fig. 3D. Wild-type Sic1 yields approximately 50 mg per liter of culture, whereas Sic1 containing all lysines mutated to arginine yields approximately 5 mg per liter. Recovery of Sic1 containing other lysine→arginine mutations is within this range. Proteins are stored in small aliquots at -80°.

G1-CDK and S-CDK Expression and Purification from Baculovirus-Infected Insect Cells

Baculoviruses are prepared to express the subunits of G1-CDK and S-CDK complexes in insect cells following the manufacturer's instructions (Invitrogen). High-titer stocks of viruses encoding G1-CDK proteins (GST-Cdc28HA, Cln2, Cak1, Cks1-His6) and S-CDK proteins (GST-Cdc28HA and Clb5) are generated as described for SCF^{Cdc4}.

1. Seed Hi5 insect cells (Invitrogen) onto 10-cm² plates at 2×10^7 and allow cells to adhere.

2. Add high-titer virus stocks in appropriate ratios. (For G1-CDK, 0.6 ml GST-Cdc28HA, 2.4 ml Cln2, 0.3 ml Cak1, and 0.3 ml Cks1-His6 are typically used. For S-CDK, 0.6 ml GST-Cdc28HA and 1.8 ml Clb5 are used.)

3. Incubate infected cells for 48 h at 27°.
4. Remove cells from plates and wash with PBS.
5. Resuspend cell pellets in cold Sf9 lysis buffer (25 mM HEPES, pH 7.4, 250 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 1 mM DTT, 5 mM MgCl₂, 10% glycerol) using 2 ml buffer per 10-cm² plate.
6. Sonicate briefly and pellet cellular debris. At this point, the clarified lysates can be stored indefinitely at -80° or used directly to purify G1-CDK or S-CDK.
7. Add 25 μl of glutathione-Sepharose (Pharmacia) to 1 ml of the lysate and incubate for 1 h at 4° with mixing.
8. Wash four times with Sf9 lysis buffer. (At this point, bead-bound complexes can be utilized if desired.)
9. Elute twice with 50 μl 500 mM Tris-HCl, pH 8, 100 mM glutathione, 250 mM NaCl, 0.25% Triton X-100, and 2 mM DTT. (The high concentration of Tris-HCl in the elution buffer is to prevent acidification caused by the high concentration of glutathione used. This glutathione concentration was determined empirically to be optimal for eluting these complexes off of glutathione-Sepharose.)
10. Check eluate and dialyze against 30 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM MgCl₂, 2 mM DTT, and 15% glycerol (two changes of 1 liter each). Resulting material can be concentrated if desired in a Biomax (Millipore) centrifugal device. Typically, 0.25 mg of G1-CDK and 2 mg of S-CDK are recovered per 10-cm² plate.

Purified G1-CDK is shown in [Fig. 3E](#) and S-CDK is shown in [Fig. 3F](#). Small aliquots are stored at -80°.

Phosphorylation of Sic1

Sic1 must be phosphorylated by G1-CDK for its recognition by SCF^{Cdc4} and ubiquitination by Cdc34.

1. Immobilize G1-CDK on glutathione-Sepharose beads. Usually, 10 to 15 μl of glutathione-Sepharose (Pharmacia) is used per 500 μl lysate. Incubate 1 h at 4° with mixing.
2. Wash four times with Sf9 lysis buffer. Wash three times with kinase buffer (20 mM HEPES, pH 8, 200 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT).
3. Assemble kinase reaction. To glutathione beads, add Sic1 diluted in kinase buffer to 88 μl. Several hundred picomoles of Sic1 can be added with little effect on the efficiency of phosphorylation. Add 2 μl of 100 mM ATP. (If radio-labeled Sic1 is desired, substitute 2 μl of [γ -³²P]ATP (4500 Ci/mmol) and 1 μl of 1 mM ATP instead of 2 μl of 100 mM ATP.)

4. Incubate 1 h at room temperature with mixing. Add 1 μ l 100 mM ATP and incubate another hour at room temperature.
5. Pellet beads and keep supernatant. Under these conditions, essentially all of the substrate is converted to a phosphorylated form (as judged by its change in mobility compared to unphosphorylated Sic1 by SDS-PAGE and Western blotting). See Fig. 4 for an example.

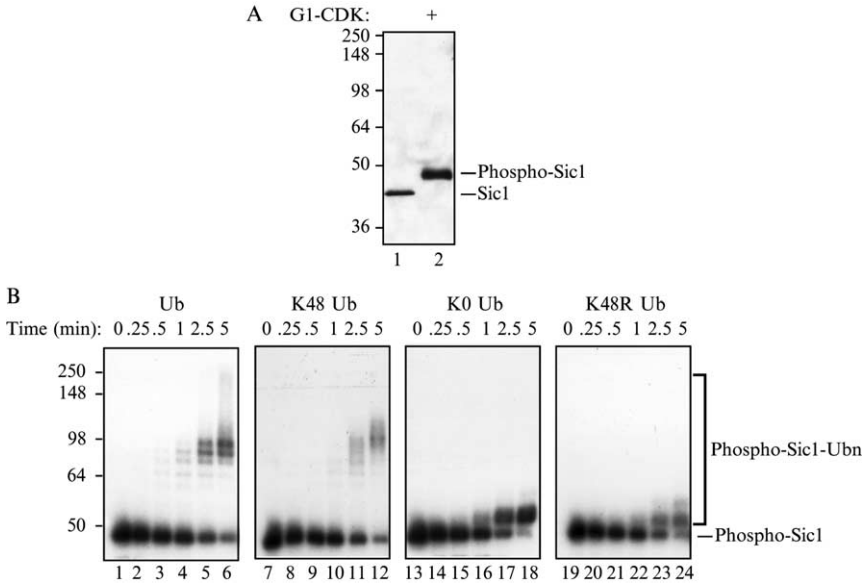


FIG. 4. Ubiquitination of Sic1 with various ubiquitin derivatives. (A) Sic1 was phosphorylated by glutathione-Sepharose-bound G1-CDK in the presence of 2 mM ATP. After incubating at room temperature for 2 h, the resulting material was analyzed by SDS-PAGE and Western blotting with antisera against the T7 tag, which is the N-terminal epitope tag on this form of Sic1. The relative position of unphosphorylated Sic1 (lane 1) and phosphorylated Sic1 (lane 2) is shown. (B) *In vitro* ubiquitination assays were performed with purified reaction components and several different ubiquitin derivatives. ³²P-labeled Sic1 (K36 only, 2.5 μ M) was premixed with SCF^{Cdc4} (100 nM) prior to addition to reactions containing Uba1 (150 nM), Cdc34 (800 nM), ATP (2 mM), and the various ubiquitin derivatives indicated (80 μ M). After mixing, aliquots were taken and added to an equal volume of sample buffer at the various times indicated. The resulting reaction products were analyzed by SDS-PAGE, followed by autoradiography. K48 Ub is ubiquitin containing all lysines mutated to arginine except lysine 48, K0 Ub lacks all lysine residues, and K48R ubiquitin has lysine 48 mutated to arginine. The relative positions of phosphorylated Sic1 and its ubiquitinated forms are indicated.

Sic1 Ubiquitination

After phosphorylation, Sic1 can be added to ubiquitination reactions containing ATP, ubiquitin, Uba1, Cdc34, and SCF^{Cdc4}. Perhaps the most difficult aspect of this assay is that multiple proteins are required and all should be titrated individually to determine optimal reaction conditions. Furthermore, buffer conditions and protein purity may have a severe impact on the “quality” of ubiquitination. The conditions described here have been determined empirically to be optimal for Sic1 ubiquitination and may serve as a starting point for substrates of other ubiquitin ligases. It is also important to note that the inclusion of NaCl in these reactions suppresses nonspecific phosphorylation-independent ubiquitination, but may reduce overall reaction efficiency.

Assemble Sic1 into a Complex with S-CDK if Desired

Binding of S-CDK to the C-terminal domain of Sic1 suppresses spurious ubiquitination on C-terminal lysines. Genetic analysis suggests that ubiquitination of Sic1 on C-terminal lysines is not physiologically relevant, and *in vitro* studies indicate that ubiquitin chains conjugated to the C-terminal domain of Sic1 do not sustain rapid degradation by the proteasome (Petroski and Deshaies, 2003), and thus we have sought to minimize this side reaction. It is probably best to empirically determine the amount of S-CDK that is needed to drive the assembly of Sic1 into heterotrimeric complexes. Alternatively, one could immobilize S-CDK on an affinity support such as on glutathione-Sepharose (described in detail earlier) and add Sic1 in saturating amounts relative to S-CDK. After washing away unbound Sic1, the eluted material from the glutathione-Sepharose should contain stoichiometric complexes of GST-Cdc28, Cib5, and Sic1.

Ubiquitination Assay

Prepare 10× ubiquitination reaction buffer (300 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 1 M NaCl, 20 mM ATP, and 20 mM DTT). Usually, reaction components are added to 10× ubiquitination reaction buffer and water to yield 1× concentration at the final volume. A typical 20- μ l reaction consists of the following components added in this order:

- 2 μ l 10× ubiquitination reaction buffer
- 13.2 μ l H₂O
- 0.5 μ l ubiquitin (80 μ M)
- 0.3 μ l Uba1 (150 nM)
- 1 μ l Cdc34 (variable concentration, >500 nM with 100 nM SCF will result in rapid and robust ubiquitin conjugation in less than 10 min)

- 1 μl SCF (variable concentration, typically 100 nM)
- 2 μl phosphorylated Sic1 +/- S-CDK (variable concentration, typically in ~20-fold excess of SCF, 2 μM).

Incubate at room temperature after gentle mixing. The amount of ubiquitinated Sic1 can be monitored over time by removing aliquots and adding to SDS-PAGE sample buffer prior to gel electrophoresis. Depending on how Sic1 is prepared, Western blotting using antisera against Sic1 or its epitope tags or autoradiography of gels containing radio-labeled Sic1 can be used to analyze the reaction products. See [Figs. 1 and 2](#) for examples of Sic1 ubiquitination assays.

Different mutant or chemically modified ubiquitins are available commercially from Boston Biochem and Affiniti. Chain-terminating forms of ubiquitin such as K0 ubiquitin or methyl ubiquitin can be used to determine how many lysine residues are targeted for ubiquitin conjugation (e.g., see [Fig. 4](#)).

Acknowledgments

M. D. P. is an associate and R. J. D. is an assistant investigator of the Howard Hughes Medical Institute. We thank R. Verma, R. Feldman, C. Correll, J. Seol, and members of the Deshaies laboratory for helpful discussions during the course of this work.

References

- Aviel, S., Winberg, G., Massucci, M., and Ciechanover, A. (2000). Degradation of the Epstein-Barr virus latent membrane protein 1 (LMP1) by the ubiquitin-proteasome pathway: Targeting via ubiquitination of the N-terminal residue. *J. Biol. Chem.* **275**, 23491–23499.
- Bloom, J., Amador, V., Bartolini, F., De Martino, G., and Pagano, M. (2003). Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. *Cell* **115**, 71–82.
- Deffenbaugh, A. E., Scaglione, K. M., Zhang, L., Moore, J. M., Buranda, T., Sklar, L. A., and Skowyra, D. (2003). Release of ubiquitin-charged Cdc34-S - Ub from the RING domain is essential for ubiquitination of the SCF(Cdc4)-bound substrate Sic1. *Cell* **114**, 611–622.
- Fajerman, I., Schwartz, A. L., and Ciechanover, A. (2004). Degradation of the Id2 developmental regulator: Targeting via N-terminal ubiquitination. *Biochem. Biophys. Res. Commun.* **314**, 505–512.
- Feldman, R. M., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91**, 221–230.
- Harlow, E., and Lane, D. (1988). “Antibodies : A Laboratory Manual.” Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Ikeda, M., Ikeda, A., and Longnecker, R. (2002). Lysine-independent ubiquitination of Epstein-Barr virus LMP2A. *Virology* **300**, 153–159.
- Kamura, T., Koepp, D. M., Conrad, M. N., Skowyra, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* **284**, 657–661.

- Mendenhall, M. D. (1993). An inhibitor of p34CDC28 protein kinase activity from *Saccharomyces cerevisiae*. *Science* **259**, 216–219.
- Nakamura, Y., Gojobori, T., and Ikemura, T. (2000). Codon usage tabulated from international DNA sequence databases: Status for the year 2000. *Nucleic Acids Res.* **29**, 292.
- Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F. B., Mendenhall, M. D., Sicheri, F., Pawson, T., and Tyers, M. (2001). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature* **414**, 514–521.
- Nugroho, T. T., and Mendenhall, M. D. (1994). An inhibitor of yeast cyclin-dependent protein kinase plays an important role in ensuring the genomic integrity of daughter cells. *Mol. Cell. Biol.* **14**, 3320–3328.
- Orlicky, S., Tang, X., Willems, A., Tyers, M., and Sicheri, F. (2003). Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell* **112**, 243–256.
- Petroski, M. D., and Deshaies, R. J. (2003). Context of multiubiquitin chain attachment influences the rate of Sic1 degradation. *Mol. Cell* **11**, 1435–1444.
- Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* **6**, 9–20.
- Reinstein, E., Scheffner, M., Oren, M., Ciechanover, A., and Schwartz, A. (2000). Degradation of the E7 human papillomavirus oncoprotein by the ubiquitin-proteasome system: Targeting via ubiquitination of the N-terminal residue. *Oncogene* **19**, 5944–5950.
- Schwob, E., Bohm, T., Mendenhall, M. D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. *Cell* **79**, 233–244.
- Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K., and Deshaies, R. J. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes Dev.* **13**, 1614–1626.
- Skowrya, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91**, 209–219.
- Skowrya, D., Koepf, D. M., Kamura, T., Conrad, M. N., Conaway, R. C., Conaway, J. W., Elledge, S. J., and Harper, J. W. (1999). Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. *Science* **284**, 662–665.
- Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G., and Deshaies, R. J. (1997a). Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science* **278**, 455–460.
- Verma, R., Feldman, R. M., and Deshaies, R. J. (1997b). SIC1 is ubiquitinated *in vitro* by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. *Mol. Biol. Cell* **8**, 1427–1437.
- Verma, R., McDonald, H., Yates, J. R., 3rd, and Deshaies, R. J. (2001). Selective degradation of ubiquitinated Sic1 by purified 26S proteasome yields active S phase cyclin-Cdk. *Mol. Cell* **8**, 439–448.
- Verma, R., Oania, R., Graumann, J., and Deshaies, R. J. (2004). Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. *Cell* **118**, 99–110.
- You, J., Cohen, R. E., and Pickart, C. M. (1999). Construct for high-level expression and low misincorporation of lysine for arginine during expression of pET-encoded eukaryotic proteins in *Escherichia coli*. *Biotechniques* **27**, 950–954.