[28] Cell-Free Ubiquitination of Cell Cycle Regulators in Budding Yeast Extracts

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Introduction

Proteolysis of key cell cycle-regulatory molecules is one method of ensuring the irreversibility of phase transitions within the eukaryotic cell cycle.[1] Degradation of such molecules is typically mediated by the ubiquitin pathway, and requires the concerted action of a complex array of proteins including ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes.[2] The action of these enzymes results in the assembly of multiubiquitin chains on the substrate, which is then targeted to the 26S proteosome where it is cleaved into small peptides.

The *CDC34* pathway in budding yeast regulates exit from G1 and entry into S phase.[1] Temperature-sensitive *cdc34* mutants accumulate at G1/S with high levels of G1 cyclin–Cdc28p kinase activity and multiple buds. Two additional *cdc* mutants (*cdc53*^{ts} and *cdc4*^{ts}) as well as certain alleles of *skp1*^{ts} arrest with a similar phenotype, and there is biochemical and genetic evidence indicating that all four gene products interact to form a complex.[3–6] *CDC34* encodes a ubiquitin-conjugating enzyme and is required for rapid degradation of several proteins including G1 cyclins.[7] Genetic analysis has revealed *cdc4*^{ts}, *cdc34*^{ts}, *cdc53*^{ts}, and *skp1*^{ts} mutants arrest at G1/S owing to accumulation of high levels of the Clb/Cdc28p inhibitor Sic1p, suggesting that Sic1p might be a substrate for the *CDC34* ubiquitination pathway.[3,4] Investigation of the mechanism of the Cdc34p-dependent ubiquitination of Sic1p thus promises to reveal the biochemical transactions governing the G1/S transition in budding yeast.

To define the mechanism of action of the ubiquitin-dependent proteolytic pathways that regulate the eukaryotic cell cycle, one needs to have

[1] R. J. Deshaies, *Curr. Opin. Cell Biol.* **7,** 781 (1995).
[2] M. Hochstrasser, *Curr. Opin. Cell Biol.* **7,** 215 (1995).
[3] E. Schwob, T. Böhm, M. Mendenhall, and K. Nasmyth, *Cell* **79,** 233 (1994).
[4] C. Bai, P. Sen, K. Hofmann, L. Ma, M. Goebl, J. W. Harper, and S. J. Elledge, *Cell* **86,** 263 (1996).
[5] A. R. Willems, S. Lanker, E. E. Patton, K. L. Craig, T. F. Nason, N. Mathias, R. Kobayashi, C. Wittenberg, and M. Tyers, *Cell* **86,** 453 (1996).
[6] N. Mathias, S. L. Johnson, M. Winey, A. E. Adams, L. Goetsch, J. R. Pringle, B. Byers, and M. G. Goebl, *Mol. Cell. Biol.* **16,** 6634 (1996).
[7] R. J. Deshaies, V. Chau, and M. Kirschner, *Eur. Mol. Biol. Org. J.* **14,** 303 (1995).

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cell-free systems that faithfully reproduce these reactions. Here, we describe in detail the methods that we use to study Cdc34p-dependent ubiquitination *in vitro*. These are most applicable to Sic1p because it is the Cdc34p substrate that we have studied most intensively.

Methods for Extract Preparation

Standard Whole-Cell Extracts

To investigate the activity of the Cdc34p ubiquitination pathway, extracts are prepared from budding yeast cells synchronized in the G1 phase of the cell cycle, because genetic analysis implies that the Cdc34p pathway is active during late G1 phase. Cells are synchronized by depriving them of G1 cyclins using a strain deleted for all three G1 cyclin (*CLN*) genes and containing an integrated copy of *CLN3* expressed from the *GAL1* promoter.[8] Although these cells proliferate on galactose medium (*GAL1* promoter on), they arrest growth prior to START in G1 phase when they are shifted to glucose medium (*GAL1* promoter off).

Cells (*cln1,2,3-Δ GAL-CLN3*) are grown to an optical density at 600 nm (OD_{600}) of 0.5 at 24 or 30° in a medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) galactose. Expression of *CLN3* is extinguished by harvesting the cells, resuspending them in repressing medium [same as the preceding growth medium, except 2% (w/v) dextrose is substituted for galactose], and culturing them for ~1.5 cell doubling times at 24 or 30°. At this point, more than 95% of the cells have arrested in G1 phase as unbudded cells. Arrested cells are harvested in a GSA rotor (Sorvall, Norwalk, CT) (5 min at 5000 rpm), transferred to a disposable 50-ml screw-cap conical centrifuge tube, and washed twice with 50 ml of ice-cold water. The tube containing the washed cell pellet is pierced at the bottom with an 18-gauge needle and the cellular pellet is extruded into liquid nitrogen by forcing it through the needle hole with the pestle of a disposable 60-ml syringe. The frozen cell paste, which resembles either popcorn or thin strings depending on its water content, can be stored indefinitely at −80° prior to use.

To prepare the extract, the frozen cell paste is ground vigorously for a total of 15 min in a mortar and pestle prechilled with liquid nitrogen. Liquid nitrogen is added and allowed to evaporate every 45–60 sec to ensure that the cell paste remains frozen. The ground cell powder is transferred to a tube and thawed by adding 0.5 vol of cold YEB buffer [30 m*M* HEPES (pH 7.2), 100 m*M* potassium acetate, 1 m*M* EDTA, 1 m*M* $MgCl_2$, 10%

[8] F. R. Cross, *Mol. Cell. Biol.* **10,** 6482 (1990).

(v/v) glycerol, 2 m*M* dithiothreitol (DTT), 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), pepstatin (10 μg/ml), and leupeptin (10 μg/ml)], supplemented with an additional 200 m*M* potassium acetate. (*Note:* It is important to prechill implements that are used to manipulate the frozen cell powder to prevent the powder from sticking.) After the powder has completely thawed, the resulting extract is incubated for an additional 15 min on ice and then centrifuged at 50,000 rpm for 15 min at 4° [Beckman (Palo Alto, CA) TLA 100.3 rotor]. The supernatant fraction is desalted on a Sephadex G-25 spin column preequilibrated with YEB and recentrifuged at 50,000 rpm for 10 min at 4° (Beckman TLA 100.3 rotor). Small aliquots of this concentrated (30–40 mg/ml) whole-cell extract are quick frozen in liquid nitrogen and stored at −80°.

Modifications to Standard Procedure

Spheroplast Extract. Extracts prepared from intact yeast cells, using the preceding growth regimen and buffers, consume ATP rapidly; an ATP-regenerating system with 1 m*M* ATP, 15 m*M* creatine phosphate, and creatine phosphokinase (50 μg/ml) is exhausted within 7 min at 24° at an extract concentration of 25 mg/ml. The lifetime of the ATP-regenerating system is increased severalfold by enzymatically removing the cell wall prior to lysis, suggesting that cell wall acid phosphatase may contribute prominently to the rapid destruction of ATP. Although we have not pursued this issue further, the use of phosphatase-deficient strains, alkaline extraction buffers, or phosphate buffer salts might help alleviate this problem. To prepare spheroplast extracts, cells grown and harvested as described above are resuspended in 100 ml of 0.1 *M* Tris–SO_4 (pH 9.2)–10 m*M* DTT, incubated for 15 min at 24°, harvested by centrifugation (Sorvall SS34; 5000 rpm for 4 min at 4°), washed with 100 ml of ice-cold water, and recentrifuged. Cell walls are digested by resuspending the cell pellet in 100 ml of SB [SB: 1 *M* sorbitol, 50 m*M* Tris–HCl (pH 7.65), 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$] and adding 1.1 mg of lyticase (Sigma, St. Louis, MO) per gram wet weight of yeast cells. After ~20–30 min of incubation at 30° (spheroplast formation is allowed to proceed until at least 90% of cells lyse on dilution in water), spheroplasts are pelleted by centrifugation (Sorvall SS34 rotor; 6000 rpm for 6 min at 4°), washed once with 100 ml of SB, and recentrifuged. The washed spheroplast pellet is resuspended in 40 ml of SB and 20 ml of spheroplast suspension is underlayered with 20 ml of HSB (HSB: SB adjusted to 1.9 *M* sorbitol) in each of two 50-ml centrifuge tubes. Spheroplasts are sedimented through the HSB cushion (Sorvall HB-4 rotor; 8000 rpm for 8 min at 4°) and the pellet is resuspended carefully in 0.5 vol of 2.5×

YEB, yielding a thick slurry of spheroplasts. This slurry is dripped into liquid nitrogen, and the frozen spheroplast kernels are ground in a mortar and pestle as described for intact cells in the preceding section.

Although spheroplast extracts are somewhat more active than whole-cell extracts for reconstitution of Cln2p ubiquitination,[7] we have typically used whole-cell extracts for reconstitution of Sic1p ubiquitination because they are easier to prepare and they sustain efficient ubiquitination of Sic1p. In contrast, Zachariae and Nasmyth[9] have reported that preparation of extracts from spheroplasts is important to observe reconstitution of cyclin B ubiquitination.

Glass Bead Extract. Extracts prepared by disrupting whole cells with glass beads in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK) also sustain Cdc34p-dependent ubiquitination of Sic1p. We have not quantitatively compared the activity of these extracts with that of extracts prepared by the standard method described above.

DEAE-Fractionated Extract. To prepare extracts that are nearly devoid of Cdc34p and partially depleted of ubiquitin, we have employed a simple, single-step fractionation procedure. Standard whole-cell extract is prepared as described above, up through the first ultracentrifugation step. Clarified extract (4-ml total volume) is supplemented with 40 μl of 200 m*M* magnesium acetate, 8 μl of 1 *M* $CaCl_2$, 4 μl of RNase (10 mg/ml), 8 μl of DNase (10 mg/ml), 4 μl of hexokinase (900 U/ml, type C-301; Sigma), and 68 μl of 50% (w/v) glucose, incubated for 35 min at 16°, and then dialyzed for 2 hr against 200 vol of 25 m*M* HEPES (pH 7.6)–25 m*M* NaCl (buffer A). The incubation with hexokinase and glucose is performed to encourage disassembly of multiubiquitin conjugates. Immunoblotting with anti-ubiquitin serum, however, indicates that most high molecular weight ubiquitin conjugates are not disassembled during this incubation. Dialyzed extract (100 mg) is centrifuged at 50,000 rpm for 10 min at 4° (Beckman 100.3 TLA rotor), diluted to 25 ml with buffer A containing 2 m*M* DTT, 1 m*M* PMSF, and pepstatin and leupeptin (1 μg/ml each), and applied to a 5-ml DEAE-Sepharose column equilibrated with buffer A. The column is then washed sequentially with 2 column volumes each of buffer A, buffer A plus 50 m*M* NaCl, buffer A plus 225 m*M* NaCl, and, finally, buffer A plus 500 m*M* NaCl. The 250 m*M* NaCl eluate is concentrated by either ammonium sulfate precipitation (80%) or centrifugation in Centricon 10 units (Amicon, Danvers, MA) to a final protein concentration of 30–40 mg/ml. Concentrated 0.25 *M* fraction is dialyzed overnight against YEB, frozen in small aliquots in liquid nitrogen, and stored at −80°.

[9] W. Zachariae and K. Nasmyth, *Mol. Biol. Cell* **7,** 791 (1996).

Preparation of Substrate

Preparation of Wild-Type Transcription Templates

SIC1 is cloned downstream of the T7 RNA polymerase promoter in either a pET (Novagen, Madison, WI) or pGEM (Promega, Madison, WI) vector. *In vitro* transcription and translation reactions are set up according to manufacturer (Promega) instructions. The pET vector yields superior results. Sic1p and Cln2p are also expressed *in vitro* from polymerase chain reaction (PCR)-amplified DNA templates. In cases in which there is no T7 polymerase promoter upstream of the *SIC1* template, the PCR 5′ primer encodes the minimal T7 promoter element. Sequence requirements for efficient transcription of T7 promoters are discussed in Milligan *et al.*[10] Yields of transcript are maximal if the first three bases following the T7 polymerase core recognition sequence are purines (GGG is best). The 3′ half of the 5′ primer typically has at least 18–20 bp of homology to the template. A typical 5′ oligodeoxynucleotide used to amplify a transcribable *SIC1* PCR product is as shown [the asterisk (*) indicates the site of transcript initiation]:

gaattc	taatacgactcactata	* **g**gatcc	atg . . .
5′ clamp	core T7 promoter	purine-rich spacer	coding sequence

The second PCR primer is designed to be complementary to the 3′ end of *SIC1* [short 3′ untranslated region (UTR)] or to sequences within the vector several hundred bases downstream of the *SIC1* open reading frame (ORF) (long 3′ UTR). The PCR products containing the T7 promoter are successfully transcribed via standard procedures either without purification or following removal of nucleotides, primers, and *Taq* polymerase via gel electrophoresis or column chromatography (QiaEx; Qiagen, Chatsworth, CA).

Preparation of Mutant Transcription Templates

The ability to produce proteins from PCR products containing a T7 polymerase promoter makes it easy to map functional domains by either deletion or point mutagenesis. The simplest mutations to make are deletions of the N and C termini. N-terminal deletions are created by synthesizing primers that contain the T7 promoter followed by an ATG and sequences from the desired starting points of the ORF. C-terminal deletions are created by synthesizing reverse-complement primers that encode a stop

[10] J. F. Milligan, D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck, *Nucleic Acids Res.* **15**, 8783 (1987).

codon followed by sequences from the desired termination points of the ORF. We typically synthesize oligodeoxynucleotides with 6–9 bases preceding the termination anticodon, but we have not tested the effect of this short 3′ UTR on translation.

Making point mutations and internal deletions requires a two-step PCR amplification. In the first step, a terminal segment of the target gene is amplified using either the normal 5′ or 3′ primer in conjunction with a mutagenic oligodeoxynucleotide. The mutagenic primer should be designed mindful of the propensity of *Taq* polymerase to incorporate a nontemplated A at the 3′ end of PCR products. The product of this first PCR is purified by gel electrophoresis, and is then used as a "bridge" oligodeoxynucleotide in a second PCR containing in addition the normal 5′ and 3′ primers, and a plasmid template deleted for either the 5′ or 3′ end of the target ORF (Fig. 1). This terminal deletion prevents the ORF from being amplified by the normal 5′ and 3′ primers, thereby rendering the PCR dependent on the bridge primer. Supercoiled plasmid should be used as template, because a linearized template (for example, one generated by cleavage within the ORF) may be extended using the bridge primer as a template, yielding a wild-type full-length template that can then be amplified by the terminal primers. To evaluate the specificity of the bridge PCR, we routinely perform a battery of controls to verify that efficient amplification depends on the truncated template, bridge primer, 5′ primer, and 3′ primer. Where possible, mutants are engineered to allow rapid verification of the mutant PCR product by restriction digest. When mutated templates have been sequenced, the predicted sequences have been obtained.

The value of this method is that it allows many mutations to be rapidly screened for their effects on the function of the ORF. Because the PCR products are directly transcribed and translated without intervening cloning steps, the low rate of misincorporation by *Taq* polymerase is inconsequential for most applications.

In Vitro Translation

Both rabbit reticulocyte and wheat germ lysates efficiently sustain synthesis of radiolabeled, ubiquitination-competent Sic1p and Cln2p, although substrates produced in wheat germ lysate are of higher specific activity. Translation reactions are terminated by the addition of cold methionine to 1 m*M* and cycloheximide to 100 μg/ml to minimize background incorporation of [^{35}S]methionine in unfractionated yeast extracts. Translation of messages derived from PCR-amplified DNA templates with a short 3′ UTR often yields minor artifact bands of higher molecular weight than expected, especially in wheat germ lysate. These artifact bands are probably covalently

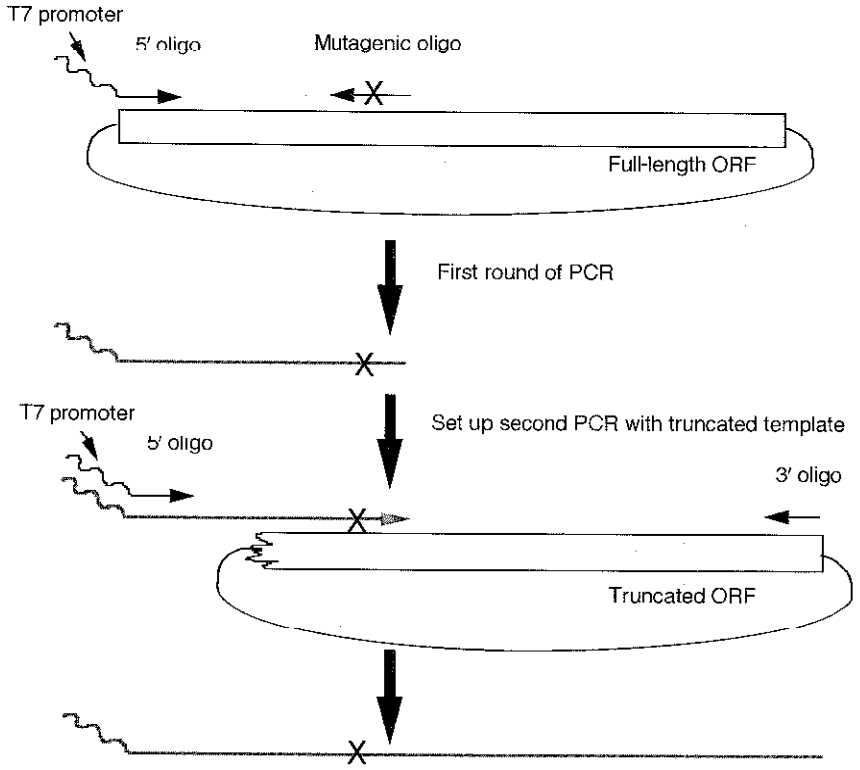

FIG. 1. Preparation of mutant transcription templates by PCR. The preparation of mutant DNA transcription templates by PCR is diagrammed. In the first PCR, a mutated N-terminal coding segment of the target open reading frame (ORF) is amplified from a template containing the full-length ORF using a T7 promoter-bearing 5′ PCR primer and a mutagenic 3′ PCR primer. The resulting PCR product is purified, and used as a bridge in a second PCR reaction containing the 5′ primer used above, a primer complementary to the 3′ end of the ORF, and an ORF-containing closed circular plasmid template that lacks the sequences complementary to the T7 promoter-bearing 5′ PCR primer.

linked protein–tRNA species that arise from inefficient termination of translation. The appearance of these artifact bands is reduced by either (1) using templates with a long 3′ UTR, (2) performing translations in reticulocyte lysate, or (3) adding puromycin (to 5 m*M*) instead of cycloheximide at the end of the translation reaction.

Production of Radiolabeled Substrate in Escherichia coli

For analytical experiments (nanogram scale), *in vitro* translation generates sufficient amounts of substrate for ubiquitination studies. For experi-

ments requiring larger amounts of substrate, radiolabeled protein is produced by expression in bacteria, using methods similar to those described by Tabor.[11] A stationary-phase culture of *Escherichia coli* cells transformed with a plasmid encoding a maltose-binding protein–Sic1p–mycHis$_6$ hybrid protein (MBP–Sic1p–MH$_6$) is diluted into 100 ml of fresh Luria broth (LB) medium and grown to an OD_{600} of 0.4. Cells are harvested, washed twice with M9 medium supplemented with a 0.005% 18-amino acid mix lacking cysteine and methionine, and resuspended in same to an OD_{600} of 0.4. After 45 min of growth at 30°, expression of MBP–Sic1p–MH$_6$ is induced by adding isopropylthiogalactoside (IPTG) to 0.4 m*M*. Following a 15-min induction, radiolabeling is initiated by the addition of Tran^{35}S label (1303 Ci/mmol; ICN, Costa Mesa, CA) at a 10-μCi/ml final concentration. Additional label (same final concentration) is added at 5, 10, and 15 min following the initiation of radiolabeling. Twenty-five minutes after the start of the labeling period, the labeled cells are harvested, washed with 25 m*M* Tris (pH 7.5)–100 m*M* NaCl to remove unincorporated label, suspended in 1 ml of wash buffer, and frozen. Radiolabeled MBP–Sic1p–MH$_6$ protein is subsequently purified from extracts of radiolabeled cells by successive chromatography on Ni^{2+}-NTA agarose (Qiagen) and amylose resins (New England Biolabs, Beverly, MA) according to supplier instructions. As can be seen in Fig. 2, two consecutive rounds of affinity purification yield radiochemically pure hybrid protein with an estimated specific activity of at least 3×10^5 cpm/μg. Neither amylose (data not shown) nor Ni^{2+}-NTA affinity chromatography (Fig. 2, lane 4) is sufficient to obtain radiochemically pure substrate. The use of two tags flanking Sic1p not only allows for efficient recovery of highly purified protein, but also eliminates contamination of the final preparation by breakdown products (Fig. 2, lanes 6 and 7). When the cloned gene is expressed from a T7 promoter, the protein can be selectively labeled by inducing expression of T7 RNA polymerase and subsequently inhibiting the host cell RNA polymerase with rifampicin. Under these conditions, radiolabel is preferentially incorporated into the target protein.[11]

Preparation of Other Reaction Components

Cdc34p has been purified from *E. coli* as described[12] and dialyzed against 20 m*M* HEPES (pH 7.2), 100 m*M* potassium acetate, 2 m*M* magnesium acetate, 2 m*M* DTT, 0.2 m*M* EDTA, and 40% (v/v) glycerol. Glutathione

[11] S. Tabor, "Current Protocols in Molecular Biology," Vol. 2, p. 16.2.5. Greene Publishers–Wiley Interscience, New York, 1995.

[12] A. Banerjee, L. Gregori, Y. Xu, and V. Chau, *J. Biol. Chem.* **268,** 5668 (1993).

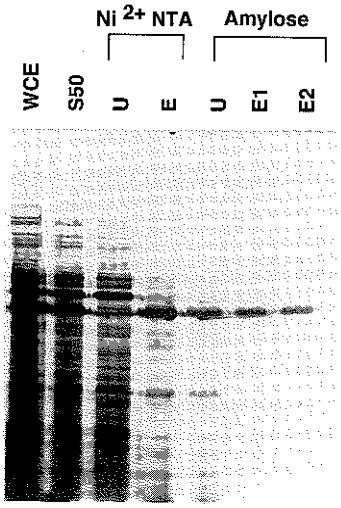

FIG. 2. Expression and purification of radiolabeled MBP–Sic1p–mycHis$_6$. *Escherichia coli* BL21+pLysS cells expressing MBP–Sic1p–MH$_6$ were pulse labeled with Tran^{35}S label as described in text, and cell extracts were prepared by freeze–thawing. Equivalent portions of each fraction were evaluated by SDS–PAGE and autoradiography. WCE, Whole-cell extract; S50, supernatant of 50,000-rpm centrifugation step prior to affinity chromatography; U, unbound fraction; E, eluate.

S-transferase (GST)–Cln2p and GST–Clb2p have been expressed in *E. coli*, purified following a procedure similar to that described by Kellogg *et al.*,[13] and dialyzed against 100 m*M* potassium acetate, 20 m*M* HEPES (pH 7.2), 2 m*M* DTT. Bovine ubiquitin (Sigma) is suspended at 20 mg/ml in 20 m*M* HEPES (pH 7.2), 2 m*M* DTT. All proteins are subaliquoted, flash frozen in liquid nitrogen, and stored at −80°.

Conditions for *in Vitro* Ubiquitination Reactions

Ubiquitination in Whole-Cell Extracts

Radiolabeled Sic1p or Cln2p (prepared by any of the methods described above) is incubated in a 10-μl reaction mixture containing yeast extract (100 μg total) plus YEB to a final volume of 5 μl, 1 μl of 10× ATP mix [10 m*M* ATP, 350 m*M* creatine phosphate, 20 m*M* HEPES pH 7.2, 10 m*M* magnesium acetate, and creatine kinase (500 μg/ml)], 1 μl of 10×

[13] D. R. Kellogg, A. Kikuchi, T. Fujii-Nakata, C. W. Turck, and A. W. Murray, *J. Cell Biol.* **130,** 661 (1995).

reaction buffer [50 m*M* magnesium acetate, 10 m*M* DTT, 5 m*M* PMSF, and pepstatin and leupeptin (100 μg/ml each)], and a 3-μl total volume of GST–cyclin (100 ng), Cdc34p (100 ng), and ubiquitin (10 μg). Reactions were incubated at 24° for 15 min, quenched by the addition of 3.5 μl 4× SDS–PAGE sample buffer, boiled, and evaluated by SDS gel electrophoresis and autoradiography. Longer incubations (up to 60 min) resulted in the gradual disappearance of all forms of Sic1p in an ATP and GST–Cln2p dependent manner. We have not rigorously tested whether the apparent destruction of Sic1p in whole-cell extract is mediated by the 26S proteasome.

Ubiquitination in DEAE-Fractionated Extracts

Reactions are set up in a total volume of 10 μl and analyzed as described above, except that whole-cell extract is substituted by 100 μg of 0.25 *M*

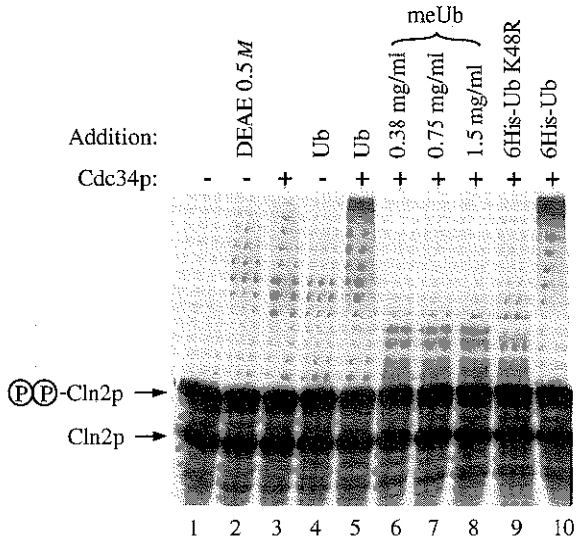

FIG. 3. Cdc34p-dependent ubiquitination of Cln2p in DEAE-fractionated extract. Spheroplast extract was prepared from a *cln1,2,3-Δ GAL-CLN3* strain and fractionated on DEAE resin as described in text. The 0.25 *M* NaCl eluate (100 μg) was mixed with 10× reaction buffer, 10× ATP mix, GST–Cln2p, ±100 ng Cdc34p, and the indicated additions in a 10-μl total volume. Lane 2 (DEAE, 0.5 *M*), 7.5 μg of yeast extract that eluted from DEAE resin between 0.25 and 0.5 *M* NaCl; lanes 4 and 5 (Ub), 5 μg of ubiquitin; lanes 6–8 (meUb), methylated ubiquitin; lane 9 (6His-UbK48R), 10 μg of ubiquitin with Lys-48 substituted by arginine, and tagged with a hexahistidine epitope at the N terminus. Both methylated and K48R ubiquitin are unable to sustain the assembly of multiubiquitin chains. [A. Hershko and H. Heller, *Biochem. Biophys. Res. Commun.* **128,** 1079 (1985); M. Hochstrasser, M. J. Ellison, V. Chau, and A. Varshavsky, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4606 (1991).]

DEAE eluate. Reactions conducted with the 0.25 *M* DEAE fraction differ from those conducted with whole-cell extract in two important respects: First, in contrast to whole-cell extracts, the formation of ubiquitin conjugates can be followed for 60 min at 24° with no loss of signal due to degradation. Second, ubiquitination of Sic1p (our unpublished data, 1997) and Cln2p is strongly stimulated by the addition of purified Cdc34p (Fig. 3; compare lanes 1 and 3 with lanes 4 and 5), because endogenous yeast Cdc34p binds tightly to DEAE resin and elutes primarily in the 0.5 *M* NaCl fraction (Fig. 3, lane 2). Other than Cdc34p, all of the other factors required for efficient ubiquitination of Sic1p bind to and elute from DEAE resin between 0.1 and 0.25 *M* NaCl. Even though ubiquitin is expected not to bind DEAE resin, the 0.25 *M* NaCl eluate used in our experiments is contaminated with low levels of ubiquitin. Nevertheless, exogenously added ubiquitin stimulates substrate ubiquitination (Fig. 3, compare lanes 3 and 5), and derivitized (or epitope-tagged) ubiquitin can be efficiently incorporated into substrates (Fig. 3, lanes 6–10). In contrast to results obtained from the reconstitution of IκB ubiquitination in HeLa cell extracts,[14] Cdc34p-dependent ubiquitination of Cln2p or Sic1p in whole-cell or fractionated yeast extracts is observed in the absence of any special inhibitors such as peptide aldehyde (proteasome inhibitor), ubiquitin aldehyde (ubiquitin isopeptidase inhibitor), and okadaic acid or calyculin A (phosphatase inhibitors). Whereas both Cln2p and Sic1p are ubiquitinated in a Cdc34p-dependent manner in both whole-cell and DEAE-fractionated extracts, Sic1p is a much better substrate. Depending on the batch of extract and the exact experimental conditions, we typically observe ubiquitination of 50–80% of the input molecules for Sic1p, and 5–20% for Cln2p.

Concluding Remarks

We describe a method that reconstitutes Cdc34p-dependent ubiquitination of Sic1p and Cln2p in whole-cell and fractionated yeast extracts. Despite the sophisticated genetic analysis of ubiquitin pathways that has been performed in budding yeast,[2] little is known about the biochemistry of these pathways because of a lack of *in vitro* systems that faithfully reconstitute ubiquitination of physiologically relevant substrates. Successes in the reconstitution of the Cdc34p[7] and anaphase-promoting complex[9] ubiquitination pathways open the door to a detailed mechanistic investigation of these pathways. Yeast biologists interested in ubiquitin-dependent proteolysis and cell cycle control can now add biochemical reconstitution to their repertoire of sophisticated molecular and genetic techniques.

[14] Z. Chen, J. Hagler, V. J. Palombella, F. Melandri, D. Scherer, D. Ballard, and T. Maniatis, *Genes Dev.* **9,** 1586 (1995).