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The Ubc3 (Cdc34) Ubiquitin-Conjugating Enzyme Is Ubiquitinated and Phosphorylated In Vivo

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The transition from G_1 to S phase of the cell cycle in *Saccharomyces cerevisiae* requires the activity of the Ubc3 (Cdc34) ubiquitin-conjugating enzyme. *S. cerevisiae* cells lacking a functional *UBC3 (CDC34)* gene are able to execute the Start function that initiates the cell cycle but fail to form a mitotic spindle or enter S phase. The Ubc3 (Cdc34) enzyme has previously been shown to catalyze the attachment of multiple ubiquitin molecules to model substrates, suggesting that the role of this enzyme in cell cycle progression depends on its targeting an endogenous protein(s) for degradation. In this report, we demonstrate that the Ubc3 (Cdc34) protein is itself a substrate for both ubiquitination and phosphorylation. Immunochemical localization of the gene product to the nucleus renders it likely that the relevant substrates similarly reside within the nucleus.

The phenotypes of various cell division cycle (*cdc*) mutants of *Saccharomyces cerevisiae* suggest a complex array of interactions among the corresponding gene products leading to DNA replication and other early functions of the cell cycle (for reviews, see references 13 and 35). The determinative event termed Start initiates a series of processes under joint control of the $p34^{CDC28}$ protein kinase and the cyclin-like products of the *CLN1*, *CLN2*, and *CLN3* genes. Cells bearing *cdc4*, *cdc34*, and *cdc53* mutations execute the Start functions but fail to proceed into S phase. Various specific functions, including bud emergence, spindle pole body duplication, and activation of Mlu cell cycle box-dependent transcription events, take place in these mutants, but the cells fail to proceed with bud maturation, spindle formation, transcriptional activation of the histone genes, or nuclear DNA replication itself (7, 9, 21, 23, 53). Furthermore, the transient sequestration of the Swi5 and Cdc46 proteins within the nucleus that normally occurs only at the time of Start becomes aberrantly persistent in *cdc4* cells (22, 33).

Molecular characterization of the *UBC3* (*CDC34*) gene has revealed a striking similarity between the deduced amino acid sequence of its gene product and that of the Ubc2 (Rad6) enzyme as well as other members of a growing family of ubiquitin-conjugating (E2) enzymes (12, 17). At least 10 distinct ubiquitin-conjugating enzymes have already been identified in *S. cerevisiae* (for a review, see reference 25), and the functional similarity of the Ubc3 (Cdc34) enzyme to other members of this family has been established: bacterially expressed Ubc3 (Cdc34) protein, in the presence of the ubiquitin-activating enzyme (E1) and ATP, shares with the Ubc2 (Rad6) enzyme the ability to monoubiquitinate histones H2A and H2B in vitro (17, 26). In addition, it has recently been demonstrated that both the Ubc2 (Rad6) and Ubc3 (Cdc34) enzymes can catalyze the addition to substrate proteins of a polyubiquitin structure similar to that which is known to cause targeting of such substrates for degradation via the ubiquitin-dependent proteolytic pathway (3, 10, 19, 46, 50). As is often the case in proteolytic targeting systems, the activity of the Ubc2 (Rad6) enzyme toward certain substrates is likely to require the function of the E3 protein (10, 46, 50). By way of contrast, the polyubiquitinating activity of the Ubc3 (Cdc34) enzyme appears to be independent of E3 (19). Nevertheless, a reasonable working hypothesis holds that the Ubc3 (Cdc34) and Ubc2 (Rad6) enzymes both exert their cellular functions by targeting specific substrate proteins for degradation.

Although it is believed that many short-lived proteins may be destabilized via ubiquitination, few natural substrates of the ubiquitin system have yet been identified (for a review, see reference 38). Two of the best-characterized examples are phytochrome (45) and the *MATα2* repressor (24). In addition, several proteins involved in cell cycle progression (the cyclins and p53) and growth control (Fos, Myc, and Mos) appear to be targeted for proteolysis by ubiquitin-dependent mechanisms (9, 15, 36, 42). Although many proteins are selected for entry into the ubiquitin-dependent proteolytic pathway by a process that is specifically dependent on the N-terminal amino acid (2, 14), this well-characterized mechanism cannot account for all cases. In particular, the ubiquitin-dependent degradation of G_2 cyclins at the appropriate stage of the cell division cycle to mediate cell cycle progression is probably independent of the N-terminal amino acid (15), and alternative regulatory mechanisms demand analysis.

We report here that the Ubc3 (Cdc34) enzyme is itself a substrate for the type of polyubiquitin modification that is known to cause targeting of proteins for degradation. We also show that Ubc3 (Cdc34) is phosphorylated in vivo, providing yet another potential means for control of its activity. Furthermore, we demonstrate a predominantly nuclear localization of the Ubc3 (Cdc34) enzyme, suggesting that substrate molecules relevant to its cell cycle function are similarly located within the nucleus.

MATERIALS AND METHODS

Strains and genetic methods. The *S. cerevisiae* strains used in this study are listed in Table 1. Standard genetic and transformation procedures were used as described elsewhere (40). *S. cerevisiae* cultures were grown in YM-1 plus dextrose at pH 5.8 (20). For plasmid selection, cells were grown in minimal medium supplemented with the appropriate amino acids or

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TABLE 1. *S. cerevisiae* strains used

Strain	Genotype	Source
2746	*MAT***a** *leu2-3,112 his7 gal*	S. L. Johnson
FY23	*MAT***a** *ura3-52 trp1-Δ63 leu2-Δ1*	F. Winston
MGG33	*MAT***a** *ura3-52 his3-Δ1 leu2-3,112 can1*	This study
YL10	*MAT***a** *cdc34-2 trp1-Δ63 ura3-52 his3-Δ1 leu2-Δ1*	Y. Liu
YPH52	*MAT***a** *ura3-52 trp1-Δ1 his3-Δ200 lys2-801 ade2-101*	P. Hieter

bases (40). For galactose induction, cells were first grown in minimal medium supplemented with 2% raffinose and then transferred to minimal medium supplemented with 2% raffinose–2% galactose. All cultures were grown at 23°C unless noted otherwise.

Plasmid constructions. The construction of plasmids pK34-1 and pK34-2 has been described previously (17). These plasmids contain the *UBC3* (*CDC34*) gene 3′ to the *tac* promoter (allowing expression in *Escherichia coli*) inserted into the vector pKK223-3 (Pharmacia) in the expressing and nonexpressing orientations, respectively (17). Plasmid pFUS34 was constructed by ligating the 1.1-kb *Hin*dIII fragment from plasmid pK34-1 (17) into plasmid pSJ101, a derivative of YEp-51 (27, 39), after digestion with *Sal*I and *Xho*I. Klenow fragment was used to fill in the incompatible 5′ overhangs of both fragments (41).

Plasmid pYcDE2-CDC34RIΔ was constructed by ligating the 0.9-kb *Sca*I-*Eco*RI fragment of pGEM34-H/S (17) into the *Eco*RI site of pYcDE-2 (kindly provided by B. D. Hall, University of Washington) after filling in the incompatible 5′ overhangs with Klenow fragment (43). The resulting plasmid contains the transcriptional promoter of the *ADH1* gene located 5′ to *UBC3* (*CDC34*) coding sequences and a transcriptional terminator region at the 3′ end. Plasmid pATH34 was generated by ligating the 430-bp *Bam*HI-*Eco*RI fragment of pGEM34H/S (17) into pATH2 (28) after cleavage with *Bam*HI and *Eco*RI.

Preparation of anti-Ubc3 antibodies. Anti-Ubc3 antiserum was prepared in New Zealand White rabbits, using as the immunogen Ubc3 (Cdc34) protein produced in *E. coli* by the method of Koerner et al. (28). Plasmid pATH34 (see above) was transformed into *E. coli* HB101, and expression of the TrpE-Ubc3 fusion protein was induced by adding indoleacrylic acid (final concentration, 5 μM) to a logarithmically growing culture. The fusion protein was purified by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by electroelution. Antiserum was affinity-purified by adsorption to polyvinylidene difluoride-bound TrpE-Ubc3 fusion protein (30).

Gel electrophoresis, Western blotting (immunoblotting), and immunoprecipitations. Yeast cell lysates were prepared by the method of Bram and Kornberg (5), with the inclusion of the following protease inhibitors: leupeptin (2 μg/ml), pepstatin (1.5 μg/ml), aprotinin (0.75 μg/ml), phenylmethylsulfonyl fluoride (0.75 μM), benzamidine (1.5 mM), and *N*-ethylmaleimide (NEM; 10 mM; Sigma). NEM was then inactivated by addition of 10 mM L-cysteine (addition of NEM inactivates many enzymes of the ubiquitin system which contain essential thiol groups, including the E1 and E2 enzymes [for a review, see reference 12], some activities of the ubiquitin-dependent protease [34], and the family of ubiquitin hydrolases [31]). Bacterial cell lysates were prepared by standard methods (41) after induction of cells with isopropylthiogalactopyranoside (IPTG; final concentration, 1 mM). Gel electrophoresis and Western immunoblotting were performed as described by Burnette (6) except that a polyvinylidene difluoride instead of nitrocellulose membrane was used. Characterization of the antiubiquitin antibodies has been reported previously (44). Approximately 20 μg of cell lysate was loaded per gel lane for SDS-PAGE (10% gel). After the transfer was complete, the blot was incubated for 1 to 2 h in Tris-buffered saline (10 mM Tris [pH 7.4], 50 mM NaCl) plus 5% nonfat dry milk and 0.5% Tween 20. Affinity-purified anti-Ubc3 (final dilution, 1:1,000) or antiubiquitin (final dilution, 1:250) was added, and the mixture was then incubated for 1 h. After several washes in Tris-buffered saline plus 0.5% Tween 20, the primary antibodies were visualized by enhanced chemiluminescence (Amersham) as instructed by the manufacturer. All incubations were performed at 23°C. Immunoprecipitations were performed by the method of Silver et al. (49).

Labeling of yeast cells. To prepare ^{32}P-labeled yeast proteins, cells were grown in YPD or YPgalactose to mid-log phase and diluted 25-fold into 25 ml of PO_4-depleted YPD (52). After cultures achieved a cell density of 5.0×10^6/ml, 5 mCi of $^{32}P_i$ (New England Nuclear) was added to the growing culture, and cells were incubated with good aeration for an additional 30 min. Immunoprecipitations and SDS-PAGE were performed as described above. After electrophoresis, gels were fixed, dried, and exposed to film. Phosphoamino acid analysis was performed on immunoprecipitates from $^{32}P_i$-labeled yeast cell lysates, using thin-layer electrophoresis as adapted from the method of Zioncheck et al. (55).

Immunofluorescent staining and photomicroscopy. Fixation and immunofluorescent staining of *S. cerevisiae* cells were performed as described previously (4). Affinity-purified anti-Ubc3 was used at dilutions ranging from 1:50 to 1:150. The secondary antibody was affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G diluted 1:200. For DNA staining, the fluorescent DNA-binding dye 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) was added to the mounting medium at 1 μg/ml. Fluorescence microscopy was performed with a Nikon FX fluorescence microscope with a 63× oil immersion objective. The anti-Ubc3 staining and DAPI staining were visualized and photographed for the same fields by changing filter sets.

RESULTS

Characterization of the Ubc3 (Cdc34) protein. To initiate an analysis of the Ubc3 (Cdc34) protein, we generated antibodies against a TrpE-Ubc3 fusion protein expressed in *E. coli* cells as described in Materials and Methods. Use of this antiserum to probe protein extracts of wild-type yeast cells by Western immunoblot analysis revealed a principal band of protein migrating at 42 kDa (Fig. 1, lanes 1 and 2), whereas the molecular mass predicted on the basis of the *UBC3* (*CDC34*) gene sequence is 34 kDa. Evidence that the aberrant migration of Ubc3 (Cdc34) protein results from intrinsic properties of this highly acidic protein rather than from posttranslational modification is provided by the finding that the gene product expressed in *E. coli* from plasmid pK34-1 also migrates at 42 kDa (Fig. 1, lane 3). Bacterial cells containing pK34-2, which has the *UBC3* (*CDC34*) gene placed in the opposite orientation, produced no immunoreactive material under the same conditions (Fig. 1, lane 4). Verification that the 42-kDa band represents the *UBC3* (*CDC34*) gene product was obtained by fusing the coding region of the *UBC3* (*CDC34*) gene (together with 3′ flanking sequence) to the promoter segment of the highly expressed *S. cerevisiae* gene encoding galactose epime-

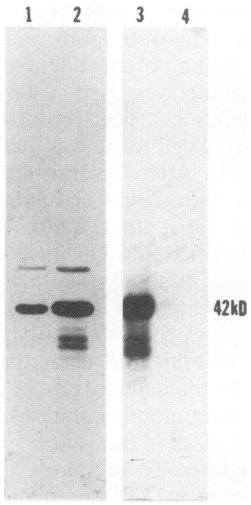

FIG. 1. Detection of the Ubc3 protein by Western blot analysis with anti-Ubc3. Lanes 1 and 2 contain total protein from wild-type yeast cells grown on YPD media; lanes 3 and 4 contain total protein from *E. coli* cells transformed with pK34-1 or pK34-2 and induced with IPTG. Lane 1, MGG33; lane 2, YPH52; lane 3, extract of HB101 containing pK34-1; lane 4, extract of HB101 containing pK34-2.

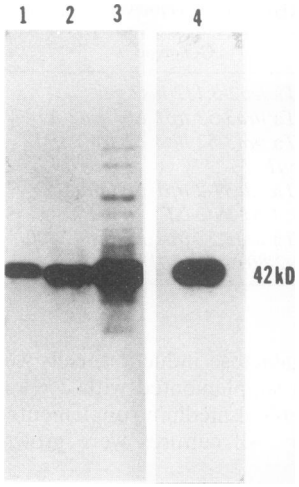

FIG. 3. Detection of multiple forms of the Ubc3 protein after immunoprecipitation with anti-Ubc3 antisera. Western blot analysis was performed on protein extracted and immunoprecipitated with anti-Ubc3 antiserum from *S. cerevisiae* FY23[pFUS34] grown under the following conditions: lane 1, raffinose; lane 2, raffinose and galactose for 6 h; lane 3, raffinose and galactose overnight; lane 4, same as lane 3 except that NEM was omitted from immunoprecipitation.

rase (*GAL10* [39]). This chimeric gene fusion, borne on plasmid pFUS34, was used to transform wild-type yeast cells. When the extracts from these yeast cells were subjected to Western blot analysis either before (Fig. 2) or after (Fig. 3) immunoprecipitation with anti-Ubc3 as described in Materials and Methods, the transformants were shown to be strongly induced for the 42-kDa protein after overnight growth on galactose medium (Fig. 2, lane 2; Fig. 3, lane 3), whereas cells grown on glucose (Fig. 2, lane 1) or raffinose (Fig. 3, lane 1) were not. A more faintly staining band with an apparent molecular mass of 52 kDa was also present in yeast cell extracts (Fig. 1, lanes 1 and 2) but was not visualized in extracts from bacteria expressing the Ubc3 (Cdc34) protein (Fig. 1, lane 3). As can be seen in Fig. 2 (lane 2), Western blot analysis of total protein from yeast cells overexpressing the *UBC3* (*CDC34*) gene with anti-Ubc3 not only revealed bands that comigrated with the 42- and 52-kDa bands seen in yeast cells not overexpressing *UBC3* (*CDC34*) but also revealed a band of greater molecular mass (Fig. 2; compare lanes 1 and 2). Overexpression of the *UBC3* (*CDC34*) gene by overnight induction with galactose (Fig. 3, lane 3), but not shorter periods of galactose inductions (Fig. 3, lane 2), also permitted visualization of proteins of even greater molecular mass that cross-reacted with anti-Ubc3 in immunoprecipitates (Fig. 3, lane 3).

FIG. 2. Detection of multiple forms of the Ubc3 protein with anti-Ubc3 antisera by Western blotting of total protein from *S. cerevisiae* 2746[pFUS34] cells probed with anti-Ubc3. The cells were grown overnight in medium containing glucose (lane 1) or raffinose and galactose (lane 2).

During the course of these experiments, it also became apparent that the bands with a molecular mass greater than 42 kDa were seen only if NEM had been added to the yeast cell extracts. If yeast cell extracts were immunoprecipitated in the absence of NEM, these bands were undetectable, even when the 42-kDa protein was greatly overexpressed (Fig. 3, lane 4). Thus, while the addition of NEM does not appear to be necessary for inactivation of a protease that acts on the 42-kDa protein, it is necessary to observe the other forms of Ubc3 (Cdc34). Since NEM inactivates many enzymes of the ubiquitin pathway, including elements of the ubiquitin-dependent protease (34) and the ubiquitin hydrolases (31), one explanation for this result is that the proteins with a molecular mass greater than 42 kDa are substrates for one or more of these enzymes.

Ubiquitination of the Ubc3 (Cdc34) protein. The presence of protein species with apparent molecular masses greater than that of the predominant 42-kDa protein and their dependence on the addition of NEM to the yeast cell extracts prior to immunoprecipitation raised the possibility that the Ubc3 protein was itself being modified by the covalent attachment of ubiquitin. To investigate this possibility, we immunoprecipitated yeast protein extracts of both wild-type and *UBC3* (*CDC34*)-overexpressing cells with anti-Ubc3 antibodies and subjected the immunoprecipitates to Western immunoblot analysis. Staining with antibodies to Ubc3 (Cdc34) in two separate experiments not only confirmed the presence of the 42- and 52-kDa bands in wild-type cells but also demonstrated additional proteins of 60 and 68 kDa in cells overexpressing *UBC3* (*CDC34*) (Fig. 4, lanes 1 and 2). When blots of the same immunoprecipitates were reacted with antiubiquitin (44), only the proteins at 52, 60, and 68 kDa were detected, with both of the two larger species being seen to stain more strongly when the *UBC3* (*CDC34*) gene was overexpressed (Fig. 4, lanes 3

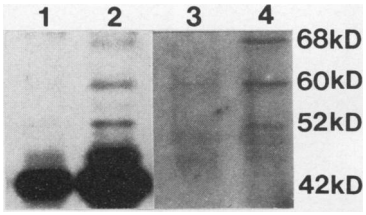

FIG. 4. Modification of the Ubc3 protein by ubiquitination. Protein extracted from *S. cerevisiae* FY23[pFUS34] and immunoprecipitated with anti-Ubc3 was subjected to Western blot analysis with anti-Ubc3 (lanes 1 and 2) or antiubiquitin (lanes 3 and 4) antibodies. Lanes 1 and 3, cells grown in 2% raffinose; lanes 2 and 4, cells first grown in 2% raffinose and then induced by growth in 2% raffinose–2% galactose overnight.

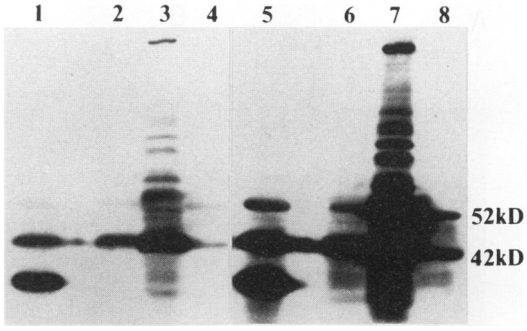

FIG. 5. The Ubc3RI truncation protein is not polyubiquitinated, as determined by Western blot analysis of total protein from FY23 cells expressing wild-type Ubc3 or Ubc3RI truncation protein and probed with anti-Ubc3. Lanes 1 and 5, FY23[pYcDe-CDC34RI] cells grown on glucose; lanes 2 and 6, FY23[pFUS34] cells grown on glucose; lanes 3 and 7, FY23[pFUS34] cells grown on galactose; lanes 4 and 8, YL10 cells grown on glucose. Lanes 5 to 8 represent a longer exposure of lanes 1 to 4.

and 4). (It is the case of course, that we may visualize not all of the proteins that coprecipitate with Ubc3 [Cdc34] but only those that are immunoreactive with anti-Ubc3 antiserum.) Our findings that the larger species are seen only when *UBC3* (*CDC34*) is overexpressed and that they are not seen if NEM is omitted provides strong evidence that these higher-mass species are modified versions of Ubc3. We cannot exclude the possibility that there are additional posttranslational modifications, but the distribution of sizes is consistent with molecular masses expected for singly (52-kDa) or multiply (60- and 68-kDa) ubiquitinated forms of the Ubc3 (Cdc34) protein. Furthermore, persistent attachment of the ubiquitin moiety following standard preparation for gel electrophoresis (boiling of the samples in buffer containing SDS and β-mercaptoethanol) indicates that this modification represents covalent attachment by an isopeptide bond rather than by the more labile thiolester bond that should be present transiently in the active site of the enzyme (3).

Haas et al. (19) have previously shown that the Ubc3 (Cdc34) protein is capable of acting in vitro to assemble a polyubiquitin structure on specific protein substrates, thus generating the type of signal that can target such proteins for degradation by the ubiquitin-dependent 26S proteasome complex (for a review, see reference 18). Furthermore, Banerjee et al. (3) have shown that the Ubc3 (Cdc34) enzyme is capable of acting on itself as one such substrate in an in vitro reaction. Since the Ubc3 enzyme can modify itself in vitro, our present demonstration of a polyubiquitinated form of the protein in cell extracts tempts us to speculate that the Ubc3 enzyme may ubiquitinate itself in vivo.

The COOH terminus is required for modification. Postulating that the unusually acidic COOH terminus of the Ubc3 (Cdc34) protein might play a role in its covalent modification, we sought to determine whether derivatives of the protein deleted for the acidic terminus were altered in modification. Truncation of the *UBC3* (*CDC34*) gene at the *Eco*RI restriction enzyme site (see Materials and Methods) should yield a protein that is missing the COOH-terminal 42 amino acid residues and therefore is reduced in mass by about 6 kDa relative to the wild-type protein. When this truncated form was overexpressed by fusion to the *ADH1* promoter in cells transformed with plasmid pYcDE-CDC34RI, the expected 36-kDa truncation derivative was indeed readily detectable (Fig. 5, lanes 1 and 5). Although this construct allowed for overexpression of the Ubc3RI truncation protein to about the same level as that seen for the wild-type protein under galactose induction (Fig. 5, lanes 3 and 7) (compare with endogenous levels of Ubc3 in wildtype [Fig. 5, lanes 2 and 6] or *ubc3* mutant [Fig. 5, lanes 4 and 8] cells), no ubiquitinated derivatives of the truncated product were detectable by Western immunoblot analysis (Fig. 5, lanes 1 and 5). Even after extended overexposure (Fig. 5, lanes 5 to 8), the only accessory bands that were detectable displayed the low abundance and molecular masses appropriate to their representing modified derivatives of the protein encoded by the wild-type genomic allele (Fig. 5, lanes 3 and 7). We conclude that sequences important for ubiquitination of the Ubc3 (Cdc34) protein, perhaps including the site(s) of ubiquitination, must reside in this COOH-terminal region.

Ubc3 is phosphorylated in vivo. The Start decision is known to be regulated by a number of protein kinases (for a review, see reference 35). To assess whether the *UBC3* (*CDC34*) function might be directly controlled in this manner, we performed experiments designed to determine whether the Ubc3 protein is phosphorylated. Cultures of strain FY23 transformed with either pFUS34 (see Materials and Methods) or pYcDE2-CDC34RIΔ were grown in PO_4-depleted YPD or YPgalactose, and $^{32}P_i$ was added to the growing cultures 30 min before the preparation of extracts. Proteins were immunoprecipitated with affinity-purified anti-Ubc3 and separated by SDS-PAGE, and autoradiograms were prepared. As can be seen in Fig. 6A, both wild-type and truncated Ubc3 are metabolically labeled with ^{32}P. Radiolabeling is seen not only for a protein of 42 kDa but also for a second band of approximately 52 kDa, suggesting that both native and ubiquitinated forms of Ubc3 are phosphorylated. As in other immunoprecipitation experiments, we cannot formally exclude the possibility that the radioactivity is incorporated into a heterologous coprecipitating protein, but the fact that two proteins of appropriate size are labeled provides strong circumstantial evidence that the label is attached to Ubc3 (Cdc34). Furthermore, when the *UBC3* (*CDC34*) gene is overexpressed, the amount of the phosphorylated 42-kDa protein is greater than that found when only the genomic product is present (Fig. 6A; compare lanes 1 and 2). This result demonstrates that at least some fraction of the Ubc3 (Cdc34) protein in dividing cells is phosphorylated. To determine which amino acid(s) is modified, the labeled immunoprecipitates were subjected to phosphoamino acid analysis (55). As shown in Fig. 6B, only phosphoserine is detected. These results suggest that Ubc3 (Cdc34), which is required for entry into S phase, may be regulated by changes in its phosphorylation state.

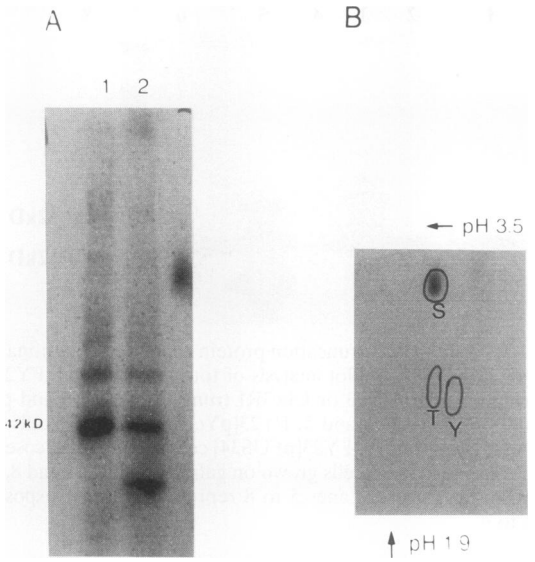

FIG. 6. Identification of Ubc3 as a phosphoprotein. (A) Cultures were labeled with $^{32}P_i$, and extracted proteins were immunoprecipitated with affinity-purified anti-Ubc3, separated by SDS-PAGE, and exposed to film. Lane 1, FY23[pFUS34]; lane 2, FY23[pYcDE2-CDC34RIΔ]. (B) Protein immunoprecipitated with anti-Ubc3 from FY23[pFUS34] labeled with ^{32}P was subjected to phosphoamino acid analysis. S, serine; T, threonine; Y, tyrosine.

Nuclear localization of the Ubc3 protein. To determine the subcellular location of the Ubc3 (Cdc34) protein, we fixed log-phase cells in 10% formaldehyde, subjected them to staining with anti-Ubc3 antibodies and with the DNA-binding dye DAPI, and then visualized the cells by fluorescence microscopy (see Materials and Methods). The most intense signal detected with anti-Ubc3 antibodies colocalized with the DAPI-stained nuclear DNA (Fig. 7). This signal was not limited to the periphery of the nucleus but could be seen to stain the nucleoplasm similarly to, but often encompassing a volume greater than, the DAPI-stained nuclear DNA. This preferential localization of Ubc3 (Cdc34) to the nucleus suggests to us that any relevant substrates of the enzyme may also be nuclear proteins. Given the possibility that autoubiquitination of Ubc3 (Cdc34) might regulate its activity in a stage-specific manner, we examined the intensity of antibody staining in the nuclei of cells representing various stages of the cell division cycle. No significant differences were seen among more than 200 cells observed, so we must conclude that any stage-specific changes of abundance or localization of the enzyme that might take place could involve only a subset of the total population of molecules.

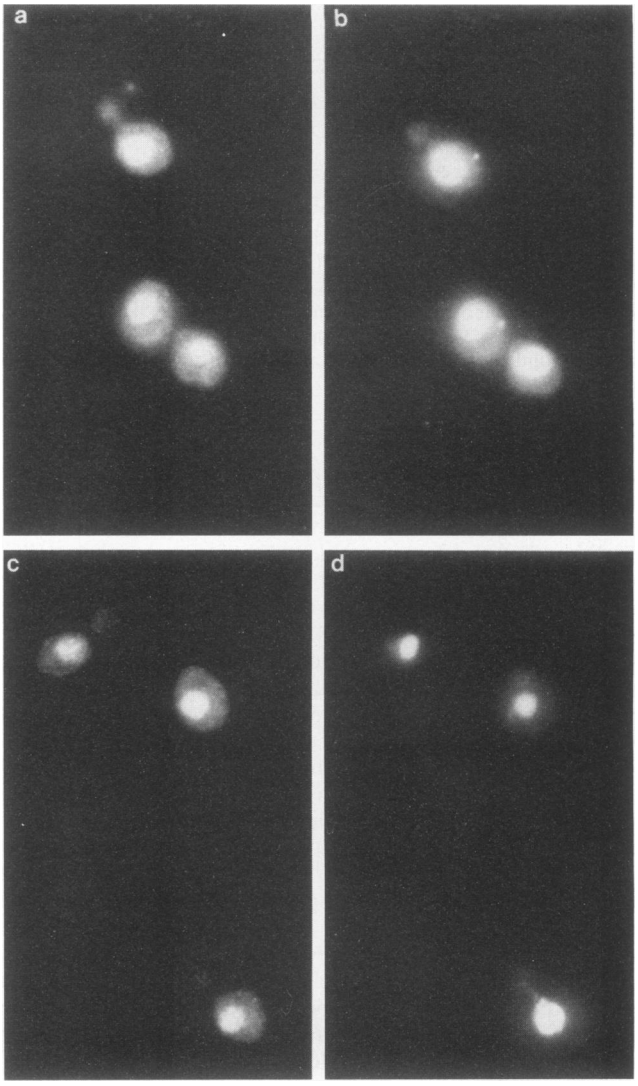

FIG. 7. Nuclear localization of Ubc3 protein. Fixed wild-type diploid cells were doubly stained with anti-Ubc3 antibodies (a and c) and with the fluorescent DNA-binding dye DAPI (b and d).

DISCUSSION

The present characterization of the Ubc3 (Cdc34) protein has shown that this ubiquitin-conjugating enzyme is itself a substrate for ubiquitin modification in vivo. Moreover, electrophoretic separation of the gene product has revealed a ladder of greater-molecular-mass derivatives, providing evidence that polyubiquitin chains of differing lengths become attached to the native protein. The formation of such ubiquitin chains is known in some cases to target substrate proteins for degradation by the ubiquitin-dependent protease system (18). It has recently been shown, in fact, that the Ubc3 (Cdc34) enzyme is capable of catalyzing the attachment of a polyubiquitin chain to itself in vitro (3). Together, these results suggest that the Ubc3 (Cdc34) ubiquitin-conjugating enzyme may be capable of promoting its own destruction in vivo.

Although the biochemical features of polyubiquitination substrates have been analyzed extensively in vitro, the present study represents one of the few cases in which this reaction has been demonstrated to have occurred in vivo (38). Our ability to detect ubiquitinated forms of Ubc3 (Cdc34) has permitted us to establish that the truncation of 42 amino acids from the COOH terminus of the polypeptide renders the protein unable to serve as a substrate in vivo. Intriguingly, this same segment of the gene product has been shown to contain a major site for autogenous polyubiquitination in vitro (3). Thus, we may now be in a position to determine the sequence requirements for recognition and modification of this substrate. Inspection of the sequences removed from the Ubc3RI truncation reveals interesting features that may affect substrate function. These features include one segment of eight contiguous aspartic acid residues and another highly charged segment that contains four lysine residues that might possibly serve as sites for

ubiquitin attachment. We are currently using in vitro mutagenesis to assess the potential roles of these regions in the ubiquitination reaction and to determine how this modification might affect the normal cellular function of the Ubc3 (Cdc34) enzyme. This portion of the protein is not essential for Ubc3 (Cdc34) activity (29, 48), for deletion of it causes no deleterious effect on cell growth. If the purpose of this ubiquitination is to target Ubc3 (Cdc34) for degradation and inactivation of Ubc3 (Cdc34) is a critical aspect of its control, then there must be other mechanisms for inactivating the enzyme rather than its physical removal; for example, the cycling of phosphorylation-dephosphorylation events (see below) may exert an additional regulatory influence over its activity.

The results presented here also demonstrate that some fraction of the *UBC3* (*CDC34*) product exists as a phosphoprotein in vivo. Most of the phosphorylated form clearly migrates near the predominant 42-kDa band. It remains to be established whether this phosphoprotein comigrates precisely with the major 42-kDa form of Ubc3 (Cdc34) or whether a minor protein band that has sometimes been seen to migrate more slowly (about 46 kDa) represents the phosphorylated form. The truncated form of Ubc3 (Cdc34) that is encoded by plasmid pYcDE2-CDC34RIΔ also becomes phosphorylated, indicating that at least some phosphorylation sites are located outside the final 42 amino acid residues that are missing from the COOH terminus in this derivative. In an effort to specify the nature of phosphorylation in Ubc3 (Cdc34), we have performed phosphoamino acid analysis on the immunoprecipitated enzyme and have detected only phosphoserine among the ^{32}P-labeled amino acids. While we cannot be certain that phosphothreonine and phosphotyrosine are completely absent, it is intriguing that phosphoserine is most readily detectable. In comparison with the sequences of the other yeast Ubc proteins, the Ubc3 (Cdc34) sequence differs in a striking manner. Upon alignment of the amino acid sequences for Ubc1, Ubc2, Ubc4, Ubc5, and Ubc8, consistent features are Lys-76 and Asp-90 (numbered according to the Ubc2 [Rad6] sequence). In Ubc3 (Cdc34), on the other hand, both of these positions are occupied by serine residues. Serine residues are also found at these positions in the human homolog of *UBC3* (*CDC34*) (37). Thus, these positions are prime candidates for sites with specific roles in Ubc3 (Cdc34) function, especially since residue 90 is very near the active-site cysteine. We are currently testing whether the specific residues present at these positions are essential for the function of Ubc3 (Cdc34) and whether they are targets of phosphorylation.

Recent findings indicate that the *CDC4/UBC3* (*CDC34*) function may act as a switch in the *S. cerevisiae* cell cycle, mediating the transition from the modified G_1 state that immediately follows Start to a later stage of G_1 that permits entry into S phase. Cells defective in the *CDC4/UBC3* (*CDC34*) step are elevated for the histone H1 kinase activity that is associated with the G_1 cyclin-like protein Cln3, while having very low levels of the Suc1-precipitable H1 kinase activity that is characteristic of the G_2/M phase transition (33, 51). A possible explanation for these findings is that polyubiquitination of various proteins responsible for controlling Start by the Ubc3 (Cdc34) enzyme targets these proteins for degradation and is thereby responsible for the transition into S phase. McKinney et al. (32) have proposed that Ubc3 (Cdc34) might target the Far1 protein, a known regulator of Start, for degradation. Regardless of which substrate is relevant to the transition into S phase, the likelihood that the Ubc3 (Cdc34) enzyme also modifies itself provides one potential mechanism for eliminating enzyme activity once it has effected its cell cycle-specific function. This presumably would permit the primary (nonautogenous) substrate(s) to begin accumulating again in preparation for the next cell cycle.

An interesting aspect of Far1 protein metabolism is the finding that in the absence of functional Ubc3 (Cdc34) enzyme, cells accumulate the phosphorylated form of the protein (32). Similarly, although the stability of total Cln3 protein is not strongly influenced by inactivation of *ubc3* (*cdc34*) (51), a hyperphosphorylated derivative of Cln3 becomes unusually abundant in *ubc3* (*cdc34*) cells, suggesting that it is the phosphorylated forms of these proteins that are most readily subjected to targeting for degradation by Ubc3 (Cdc34)-mediated polyubiquitination. It is interesting in this regard that when glucose is added to cells overexpressing galactose-induced Ubc3 (Cdc34) protein, the level of the protein remains elevated for at least 6 h (16). Thus, the total pool of Ubc3 (Cdc34) protein is quite stable, as is not expected if degradation of the entire complement of the protein were required for entry into S phase. It therefore seems likely that only a fraction of the total pool of Ubc3 (Cdc34) protein in the cell—perhaps the same fraction that has been phosphorylated—becomes subject to autogenous ubiquitination and thus is degraded.

In another aspect of this study, immunofluorescence assays have revealed preferential localization of the *UBC3* (*CDC34*) gene product to the nucleus. This finding suggests that any relevant substrate molecules (besides the enzyme itself) also reside within the nuclear compartment, where they would undergo a Ubc3 (Cdc34)-mediated polyubiquitination reaction that may target them for degradation. Assuming that polyubiquination of the enzyme itself serves a similar purpose, we have examined these stained cultures for any evidence of cell cycle-specific alterations in the abundance or localization of the Ubc3 (Cdc34) protein but have been unable to detect any variation of this sort. Our provisional conclusion is that ubiquitination does not significantly affect the turnover or distribution of the overall population of enzyme molecules. However, if it were the case that only a subset of molecules become activated to exert the essential function of *UBC3* (*CDC34*), then a large pool of latent molecules may obscure significant changes in the abundance or distribution of the activated species. More specific diagnostic methods might be required to detect distributional changes in the modified species. Finally, it must be recognized that some of the gene product resides in the cytoplasm. It remains to be explored whether this subset of molecules is actively engaged in cell cycle-specific functions of the cytoplasm that are known to be defective at *ubc3* (*cdc34*) arrest (such as bud maturation). Alternatively, the cytoplasmic protein may simply be nascent or otherwise not directly engaged in the relevant function.

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