The Yeast UBC4 Ubiquitin Conjugating Enzyme Monoubiquitinates Itself *in Vivo*: Evidence for an E2−E2 Homointeraction†

Chantelle S. Gwozd,‡ Terra G. Arnason,‡ William J. Cook,§ Vincent Chau,|| and Michael J. Ellison*,‡

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7, Department of Pathology and Center for Macromolecular Crystallography, University of Alabama at Birmingham, Birmingham, Alabama 35294, and Department of Pharmacology, Wayne State University Medical School, Detroit, Michigan 48201

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ABSTRACT: Here we report that the stress-related ubiquitin conjugating enzyme UBC4 from *Saccharomyces cerevisiae* is monoubiquitinated *in vivo*. The UBC4−ubiquitin conjugate was detected by the coexpression in yeast of epitope-tagged ubiquitin in combination with either untagged or epitope-tagged versions of UBC4. Under these conditions the UBC4 conjugate proved to be the most abundant conjugate detected. Using chemical mapping and site-directed mutation, the site of ubiquitination was localized to a single lysine (K144) near the carboxy terminus of UBC4. A second lysine within UBC4 (K64) was also identified whose mutation resulted in the loss of ubiquitination at K144. The mutation of either K64 or K144 had no obvious effect on the known *in vivo* functions associated with UBC4. In another experiment, a nonfunctional UBC4 derivative with a mutation at the active site was also found to be monoubiquitinated in a manner that depended on the expression of active UBC4. This result indicated that ubiquitin was transferred in an intermolecular reaction from one UBC4 monomer to another. Cross-linking analysis demonstrated that UBC4 monomers directly and specifically interact with one another *in vitro*. Both the *in vivo* and *in vitro* observations reported here, in combination with previous findings, support the view that interactions between ubiquitin conjugating enzymes represent a general phenomenon.

The ubiquitin (Ub)[1] conjugating enzymes or E2s are a class of eukaryotic proteins that catalyze the transfer of Ub from the Ub activating enzyme, E1, to the lysines of appropriately targeted proteins [for reviews see Finley (1992), Hershko and Ciechanover (1992), Hochstrasser (1992), Jentsch (1992), and Varshavsky (1992)]. Mutational analyses of E2 genes in yeast have revealed that these enzymes participate in a broad range of cellular processes and therefore play at least an indirect role in target protein recognition. Certain E2s also catalyze the formation of the multi-Ub chain (Chen & Pickart, 1990; Haas et al., 1991; Johnson et al., 1992), a structure whose assembly onto targeted proteins is strongly correlated with their turnover.

Several lines of evidence indicate that E2 molecules interact with one another in homo- and heterocomplexes and that in certain cases these types of interactions play an important role in E2 function. Three groups have reported for instance that certain E2s can be purified as homodimers and higher order complexes (Pickart & Rose, 1985; Haas & Bright, 1988; Girod & Viestra, 1993). Silver et al. (1992) presented genetic evidence suggesting that the cell cycle function of the CDC34 (UBC3) Ub conjugating enzyme was dependent on its interaction with itself or with the DNA repair enzyme RAD6 (UBC2). More recently, Chen et al. (1993) have found that the turnover of the yeast MATα2 transcriptional regulator is strongly correlated with the interaction of UBC6 and UBC7 and have also presented evidence for the interaction of UBC7 with itself.

The possible functions associated with E2−E2 interactions are not known. Silver et al. (1992) have proposed a model based on the interaction of one E2 molecule with another that accounts for the initiating steps in multi-Ub chain synthesis. On the other hand, Chen et al. (1993) have suggested that the ability of E2s to interact among themselves in different combinations may serve to increase the repertoire of substrate specificities found within the Ub system.

In this paper we present further evidence for the interaction of E2 monomers *in vivo*. We have found that the stress-related Ub conjugating enzyme UBC4 monoubiquitinates itself at a single lysine residue in an intermolecular reaction. This observation suggests that UBC4 interacts with itself in a specific manner and provides an important structural landmark for defining the nature of this interaction. Further evidence for UBC4 homointeractions comes from *in vitro* cross-linking studies in which UBC4 monomers can be specifically cross-linked to one another.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains. High copy *TRP1* yeast expression vectors carrying the *UBC4* gene derivatives shown in Figure 1 are identical to YEp96 (Ellison & Hochstrasser, 1991) except that the *UBC4* coding sequence replaces the

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* Corresponding author.
‡ University of Alberta.
§ University of Alabama at Birmingham.
|| Wayne State University Medical School.

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[1] Abbreviations: Ub, ubiquitin; mUb, N-terminally myc-tagged ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin protein ligase; HA, hemagglutinin; Ub-pro-β-gal, ubiquitin pro-β-galactosidase; Ub-met-β-gal, ubiquitin met-β-galactosidase; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; BS^3, bis(sulfosuccinimidyl) suberate.

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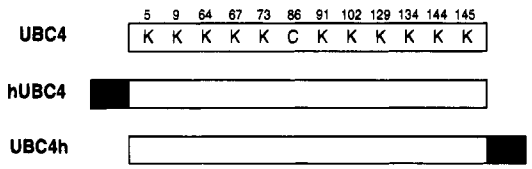

FIGURE 1: UBC4 derivatives. Open boxes represent the peptide sequence of UBC4. Black boxes represent the HA epitope. Also shown are the amino acid positions that were changed (K to R, C to A) to produce the mutated UBC4 derivatives.

Ub coding sequence. In all *UBC4* plasmids, the serine-2 codon AGC has replaced the serine-2 codon TCT found in the wild-type sequence (Seufert & Jentsch, 1990) in order to create a *Sac*I site. Lysine to arginine mutations were made by changing the appropriate lysine codon to the arginine codon AGA. In *ubc4A86*, the active site cysteine-86 codon was replaced with the alanine codon GCT. The influenza virus hemagglutinin peptide epitope sequence (Wilson et al., 1984), which will be referred to here as the HA tag, was fused to both the amino and carboxy termini of UBC4. N-Terminally HA-tagged UBC4 (hUBC4) was created by appending a sequence encoding the peptide MYPYDVPDYASLG to the methionine-1 codon of *UBC4* (peptide epitope underlined). C-Terminally HA-tagged UBC4 (UBC4h) was created by appending a sequence encoding the peptide GYPYDVPDYASLG to the last codon of *UBC4*. The coding sequence of each *UBC4* derivative was verified by DNA sequencing using an Applied Biosystems 373A DNA sequencer operated by the University of Alberta, Department of Biochemistry, DNA Synthesis and Sequencing Laboratory.

Low-copy *TRP1* plasmids carrying *UBC4* and its arginine-64 (*ubc4R64*) and arginine-144 (*ubc4R144*) derivatives under the control of the native *UBC4* promoter were constructed by positioning an expression cassette between the *Cla*I and *Eco*RI sites of pRS314 (Sikorski & Hieter, 1989). Sequences of the cassette which define the *UBC4* coding sequence and downstream *CYC1* terminator sequence (including the flanking *Cla*I site) are identical to the high-copy vectors described above. The promoter sequence of *UBC4* extends from −1 to −524 (with a G to T change at −521 to create an *Eco*RI site) and is contiguous to the *Eco*RI site of the vector.

The yeast high-copy *URA3* plasmids expressing either Ub or N-terminally myc-tagged Ub (mUb) were created by inserting the *Bam*HI/*Cla*I fragment (containing the *CUP1* promoter, *Ub* or *mUb* coding sequence, and *CYC1* terminator) from either YEp96 or YEp105 (Ellison & Hochstrasser, 1991) into YEp352 (Hill et al., 1986) between the *Bam*HI and *Nar*I sites. The high-copy *TRP1* negative control plasmid pES12 (Hodgins et al., 1992) is identical to YEp96 except for deletion of the *Ub* coding sequence. The high-copy *URA3*-pUB23P plasmid [expressing Ub-pro-β-galactosidase (Ub-pro-β-gal)] and the *URA3*-pUB23M plasmid [expressing Ub-met-β-galactosidase (Ub-met-β-gal)] have been previously described (Bachmair et al., 1986).

The pET3a-UBC4 plasmid used for the expression and purification of recombinant UBC4 from *Escherichia coli* is identical in sequence to the pET3a-CDC34 plasmids described elsewhere (Ptak et al., 1994) except that the coding sequence for UBC4 (as described above but minus its intron) replaces the coding sequence of CDC34. Details on the construction of all plasmids described here for the first time are available on request.

For Western analysis, selected high-copy *TRP1-UBC4* plasmids and the control plasmid pES12 were introduced into either of the yeast strains SUB60 (Finley et al., 1987) or MHY498 (Chen et al., 1993) in combination with either the *Ub-URA3* or *mUb-URA3* plasmids. SUB60 carries a deletion for the polyUb gene *UBI4* and has the genotype *MAT* a, *lys2-801*, *leu2-3*, *leu2-112*, *ura3-52*, *his3-Δ200*, *trp1-1*(am), *ubi4Δ::LEU2*. MHY498 (obtained from M. Hochstrasser) carries a deletion for the *UBC4* gene and has the genotype *MAT* α, *his3-Δ200*, *leu2-3*, *112*, *ura3-52*, *lys2-801*, *trp1-1*, *ubc4-Δ1::HIS3*.

For phenotype analysis, low-copy plasmids expressing either wild-type UBC4, ubc4R64, or ubc4R144 were introduced into MHY508 (obtained from M. Hochstrasser). MHY508 (Chen et al., 1993) carries a deletion for *UBC4* and *UBC5* and has the genotype *MAT* α, *his3-Δ200*, *leu2-3*, *112*, *ura3-52*, *lys2-801*, *trp1-1*, *ubc4-Δ1::HIS3*, *ubc5-Δ1::LEU2*.

Steady-state levels of Ub-pro-β-gal and Ub-met-β-gal were determined using the above three MHY508 strains cotransformed with either of the pUB23 plasmids or YEp352.

Protein Expression and Western Analysis. Cells were grown to early exponential phase at 30 °C in synthetic-defined (SD) media (Sherman et al., 1986) supplemented with selected amino acids critical for growth (for SUB60, histidine, 10 mg/L, and lysine, 40 mg/L; for MHY498, lysine, 40 mg/L, and leucine, 60 mg/L). Cultures were then diluted to 3×10^6 cells/mL in the presence of $CuSO_4$ (100 μM) to induce gene expression and were allowed to grow for two additional generations prior to harvesting. Cells were then washed in cold 10 mM $CdCl_2$ and 2 mM EDTA (to inhibit proteolysis and isopeptidase activity), followed by resuspension in electrophoresis load mix [12.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.001% bromophenol blue, 200 mM dithiothreitol (DTT)]. Samples were then boiled (10 min) and centrifuged to remove cellular debris. Sample supernatants were electrophoresed on an SDS−polyacrylamide gel [18% acrylamide, 0.09% bis-(acrylamide)]. For Figure 2, Western analysis was performed as described in Hodgins et al. (1992). All other Western analyses were performed as above except that, following anti-myc antibody treatment, filters were washed in 1× Tris-buffered saline + Tween (TBS + Tween, 10 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.05% Tween-20) three times, followed by incubation in 50 mL of TBS + Tween containing 17 μL of goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad) for 45 min at 4 °C. Filters were then washed as above. Protein bands bearing the myc epitope were visualized using enhanced chemiluminesence (ECL) (Amersham).

Chemical Cleavage Analysis. SUB60 cells coexpressing mUb and either UBC4 or C-terminally HA-tagged UBC4 (UBC4h) from high-copy vectors were grown and processed in the same way as described for Western analysis. Protein samples from approximately 1×10^8 cells were electrophoresed on an 18% polyacrylamide−SDS gel. Following electrophoresis, mUb−UBC4 and mUb−UBC4h conjugates were excised from the gel on the basis of their known migration relative to prestained molecular weight markers. Protein was electroeluted from the acrylamide slices using the procedure of Hunkapiller et al. (1983). Formic acid was added to the protein solution to 70%, and the reaction was incubated at 37 °C for 24 h. BSA was then added as a carrier, and the reaction was extracted using the methanol−

chloroform procedure of Wessel and Flügge (1984). The resulting protein precipitate was boiled in electrophoresis load mix, and Western analysis was performed as outlined above.

UBC4 Expression and Purification. The pET3a-UBC4 expression plasmid was cotransformed into the *E. coli* strain BL21 in combination with the thermally inducible T7 polymerase plasmid pGP1-2 (Tabor & Richardson, 1985). Cells were grown in LB liquid media (containing 50 μg/mL ampicillin and 40 μg/mL kanamycin) to an absorbance of 0.4 at 590 nm. Cells were washed two times in cold M9 media and then grown for 2 h at 30 °C in 25 mL of M9 media supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 12 mM glucose, 18 μg/mL thiamin, 50 μg/mL ampicillin, 40 μg/mL kanamycin, and all amino acids (40 μg/mL) except for cysteine and methionine. Cultures were transferred to 42 °C for 40 min followed by the addition of rifampicin (200 μg/mL final). Cells were incubated for 30 min at 42 °C followed by a shift to 30 °C for 20 min. *trans*-[^{35}S]-Methionine (ICN) was then added (25 μCi/mL) followed by incubation for 40 min at 30 °C. Cells were harvested by centrifugation, resuspended in 250 μL of 25% sucrose and 50 mM Tris-HCl (pH 8.0), and lysed with lysozyme as previously described (Gonda et al., 1989). Labeled UBC4 was purified using an FPLC system (Pharmacia Biotech Inc.). Clarified supernatants in 20 mM Hepes, pH 6.9, and 1 mM DTT were passed over a Mono S HR 5/5 ion-exchange column (Pharmacia) equilibrated with the same buffer. The flow through was collected and then passed over a Mono Q HR 5/10 ion-exchange column (Pharmacia), equilibrated with 50 mM Tris-HCl, pH 7.5, and 1 mM DTT, and eluted with a NaCl gradient from 0 to 1 M. Under these conditions, UBC4 eluted in essentially pure form as a major protein peak at 60 mM NaCl.

Protein Cross-Linking. Prior to cross-linking, UBC4 was dialyzed into cross-linking buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM DTT). Aliquots (40 μL) were preincubated on ice for 5 min, followed by the addition of 0.1 volume of the cross-linker BS^3 [bis(sulfosuccinimidyl) suberate (Pierce)] in cross-linking buffer. Samples were incubated an additional 30 min on ice. BS^3 was added to a final concentration of 0, 0.4, or 10 mM to reactions that contained ^{35}S-labeled UBC4 (6.6 μM, 1.6×10^4 cpm/μM) in the presence (66 μM) or absence of bovine serum albumin (BSA). Cross-linked species were detected by SDS−polyacrylamide gel electrophoresis (16%) followed by autoradiography.

Phenotype Analysis. For stress sensitivity experiments MHY508 strains were grown in SD media supplemented with uracil (40 mg/L) and lysine (40 mg/L) as described above. Appropriate dilutions of early exponential cultures were then spread onto supplemented SD plates with or without canavanine (1.5 μg/mL) and grown at 30 °C. Cultures were also streaked onto YPD plates and grown at 38 °C. For β-galactosidase experiments, cells were grown to a final density of 5×10^7 cells/mL in liquid media containing 0.7% Difco yeast nitrogen base, without amino acids, 2% galactose, 2% glycerol, 2% ethanol, and lysine (40 mg/mL). β-Galactosidase activity was measured as previously described (Reynolds & Lundblad, 1989).

RESULTS

UBC4 Is Monoubiquitinated in Vivo. In previous work we showed that the expression of mUb in yeast could be a useful tool for detecting Ub−protein conjugates formed *in vivo* (Ellison & Hochstrasser, 1991). Typically, these tagged conjugates were detected by SDS−polyacrylamide gel electrophoresis (PAGE) of total yeast protein followed by Western analysis using an anti-myc epitope antibody. Interestingly, when this analysis was applied to cells overexpressing the stress Ub conjugating enzyme UBC4 from a high-copy plasmid, a prominent band of approximately 25 kDa was detected that rivaled the levels of free mUb (Figure 2). The absence of this band in cells expressing UBC4 in combination with unmodified Ub establishes that mUb is present in this species and that the band is not another protein that cross-reacts with the anti-myc antibody. In addition, the absence of the band in cells expressing only mUb (not UBC4) demonstrates that this species is formed in a UBC4-dependent manner. On the basis of the above observations and the molecular mass of mUb (approximately 10 kDa) it can be concluded that the 25 kDa species is a mUb−protein conjugate in which mUb is coupled in a UBC4-dependent manner to another protein, of approximately 15 kDa in molecular mass.

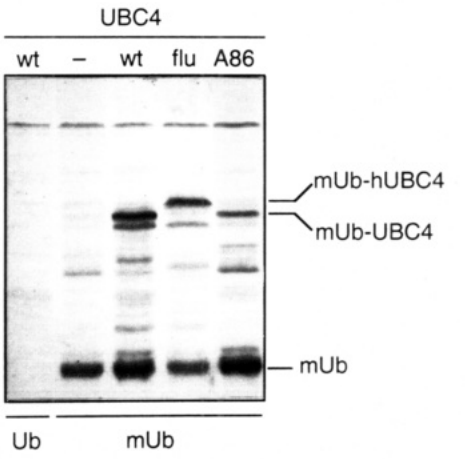

FIGURE 2: Monoubiquitination of UBC4 *in vivo*. Immunoblot of an SDS gel (probed with the anti-myc antibody) of total protein from yeast expressing either wild-type UBC4 (wt), a negative control plasmid (−), N-terminally HA-tagged UBC4 (flu or hUBC4), or ubc4A86 (A86) in combination with either ubiquitin (Ub) or N-terminally myc-tagged ubiquitin (mUb). The positions of mUb and the mUb−UBC4 and mUb−hUBC4 conjugates are shown. The assignment of molecular weights was made on the basis of migration with respect to known molecular weight markers.

It was possible that the 15 kDa component of the conjugate was an abundant substrate for UBC4-dependent ubiquitination and that the steady-state levels of the conjugate increased dramatically when UBC4 was overexpressed. Another possibility, however, was that the conjugate was composed of mUb and UBC4 itself, an idea that is consistent with both its abundance and molecular mass. We tested this idea by altering the molecular mass of UBC4 (approximately 1.4 kDa) by appending the HA peptide tag to its amino terminus. If the mUb conjugate contains UBC4, then expression of tagged UBC4 should shift the electrophoretic position of the mUb conjugate upward by a corresponding amount. If, however, UBC4 is not the target of ubiquitination, then the position of the mUb conjugate will remain unaltered. As indicated in Figure 2, the decreased mobility of the mUb conjugate from cells expressing tagged UBC4 relative to cells expressing untagged UBC4 demonstrates that the major conjugate formed under these conditions consists of a single molecule of mUb coupled to UBC4.

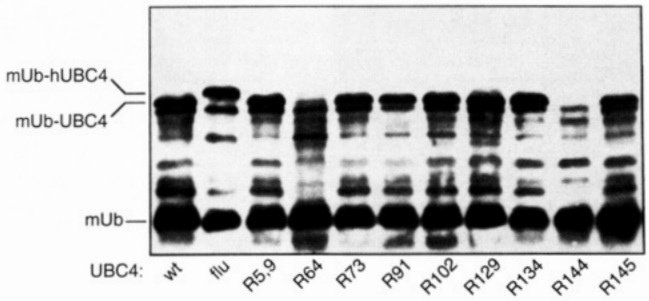

FIGURE 3: Mapping the site of UBC4 ubiquitination by mutation. Immunoblot of an SDS gel (probed with the anti-myc antibody) of total protein from yeast expressing wild-type UBC4 (wt), N-terminally HA-tagged UBC4 (hUBC4), or one of the UBC4 arginine mutants (R5−R145) in combination with N-terminally myc-tagged ubiquitin (mUb). The positions of mUb and the mUb−UBC4 and mUb−hUBC4 conjugates are shown.

It was possible that the mUb−UBC4 conjugate was actually a thiol ester intermediate formed by the transfer of mUb by the Ub activating enzyme (E1) to cysteine-86 of UBC4. This possibility seemed unlikely in view of the acknowledged instability of Ub−E2 thiol esters and in light of the fact that the protein samples were boiled in 200 mM DTT prior to electrophoresis. However, in order to definitively rule out this possibility, a UBC4 derivative that was defective in thiol ester formation was constructed in which cysteine-86 was replaced with alanine. The observation that this derivative was still targeted for ubiquitination (Figure 2) indicated that the mUb−UBC4 conjugate was not a thiol ester intermediate and that mUb was being conjugated to some other site, most probably a lysine. Significantly, this result also demonstrated that the ubiquitination of UBC4 does not occur by an intramolecular reaction mechanism in which mUb is passed from the active site of UBC4 to a site on the same molecule. Instead, the ubiquitination of UBC4 must be catalyzed by the transfer of mUb to UBC4 by another E2 molecule.

Ubiquitination of UBC4 Occurs at a Single Lysine. To determine which lysine in UBC4 is targeted for ubiquitination, a series of *UBC4* gene derivatives were created that carried lysine to arginine codon substitutions at different positions within the *UBC4* coding sequence. If a single lysine within UBC4 served as the site of ubiquitination, then mutation of this residue would result in the complete disappearance of the UBC4 conjugate. When cells expressing the various UBC4 mutants were subjected to the same analysis as described for Figure 2, it was found that lysine to arginine substitutions at either position 64 or 144 result in the disappearance of the UBC4 conjugate while lysine to arginine substitutions at other positions had no effect (Figure 3). If both positions, 64 and 144, served as targets for ubiquitination, then substitution at one position would still leave the other available for mUb attachment and would result in only a partial reduction in UBC4 conjugate levels. The complete disappearance of the UBC4 conjugate in either case, however, argues that one lysine is targeted for ubiquitination while the other lysine fulfills an indirect but essential role in the reaction.

A chemical cleavage strategy was used to discern which of the two lysines, K64 or K144, was ubiquitinated (Figure 4A). UBC4 contains two Asp−Pro peptide bonds that are sensitive to cleavage upon treatment with formic acid. One of these peptide bonds is situated between K64 and K144 at

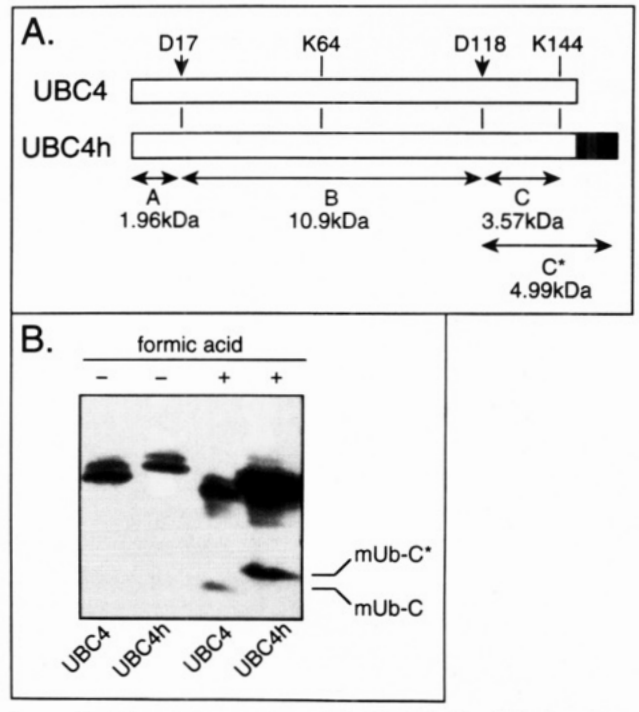

FIGURE 4: Chemical mapping of the UBC4 ubiquitination site to K144. (A) Schematic of the mapping strategy showing the two formic acid cleavage sites (D17 and D118) in UBC4 and UBC4h and the resulting fragments. (B) Immunoblot of an SDS gel (probed with the anti-myc antibody) showing the formic acid cleavage pattern of the mUb−UBC4 conjugate (UBC4) or the mUb−UBC4h conjugate (UBC4h). mUb−C and mUb−C* mark the positions of the myc-ubiquitinated forms of the C fragments illustrated in (A).

position 118. Cleavage of mUb−UBC4 with formic acid would give rise to three fragments whose molecular weights depend upon whether mUb was positioned at K64 or K144 (Ub does not contain an acid-sensitive site). By comparison of the electrophoretic cleavage pattern of purified conjugates formed from either UBC4 or C-terminally HA-tagged UBC4 (UBC4h), ubiquitination at K64 or K144 can be assigned unambiguously. The positioning of mUb at K64, for instance, would give rise to a fragment of approximately 21 kDa (fragment B + mUb) common to both the UBC4 and tagged UBC4 conjugates. The positioning of mUb at K144 would, however, give rise to a smaller fragment of approximately 14 kDa for the UBC4 conjugate which would shift to approximately 15 kDa upon addition of the tag (fragment C* + mUb). Furthermore, only those fragments coupled to mUb can be visualized using the anti-myc antibody as a probe. As seen in Figure 4B, the addition of the HA tag to UBC4 results in a corresponding increase in the molecular weight of the smallest fragment coupled to mUb. On the basis of this result and the results of Figure 3, it can be concluded that UBC4 is ubiquitinated at K144. The highest molecular weight species remaining after formic acid cleavage (Figure 4B, + lanes) are most likely the result of a single cleavage event at D17, based on their calculated molecular weights relative to molecular weight standards (not shown).

UBC4 Monoubiquitinates Itself. The observation that the active site mutant of UBC4 (ubc4A86) is ubiquitinated *in vivo* indicated that mUb was being transferred to UBC4 in an intermolecular reaction catalyzed by one of potentially any of the E2s found in yeast including UBC4 and its homologue UBC5. In principle, the involvement of either

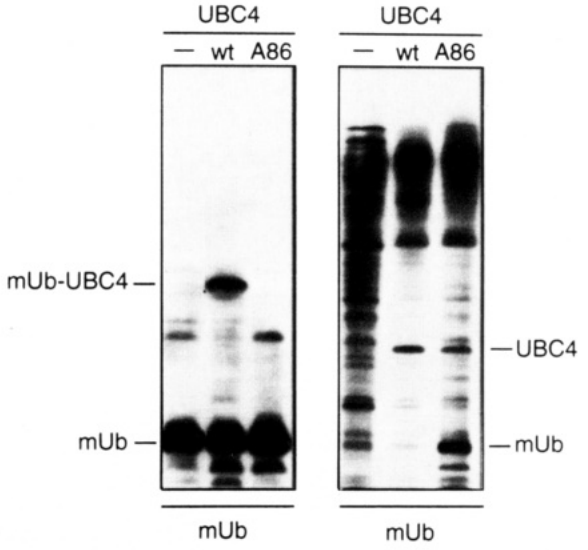

FIGURE 5: UBC4 monoubiquitinates itself in an intermolecular reaction. Total yeast protein from cells labeled with [^{35}S]methionine and expressing mUb alone (−) or in combination with UBC4 (wt) or ubc4A86 (A86). Left panel: an immunoblot of an SDS gel probed with the anti-myc antibody. Right panel: an autoradiograph of an SDS gel.

UBC4 or UBC5 in this reaction was best resolved by examining whether or not ubc4A86 was ubiquitinated in a strain of yeast deleted for both the *UBC4* and *UBC5* genes. We expected that if UBC4 is ubiquitinated by another UBC4 monomer, then ubc4A86, which can act only as an acceptor of Ub and not as a donor, would not be ubiquitinated in a *ubc4ubc5*Δ strain. In practice, however, expression of ubc4A86 in the double mutant proved to be toxic (results not shown). It is possible that the toxicity arising from ubc4A86 expression may be the result of sequestration of an essential E3. In contrast, cells expressing ubc4A86 in a strain deleted only for *UBC4* were viable. Furthermore, the results of Figure 5 illustrate that when UBC4 and ubc4A86 are expressed to similar levels in the *UBC4* deleted strain (right panel), UBC4 is ubiquitinated whereas ubc4A86 is not (left panel). On the basis of these results, we conclude that UBC4 ubiquitinates itself. The strong dependence of this ubiquitination on UBC4 activity, inspite of the presence of the *UBC5* gene, can be explained by the previous observation that, under exponential growth conditions, UBC5 expression is low when compared to UBC4 (Seufert & Jentsch, 1990).

Nonubiquitinatable ubc4 Mutants Have No Obvious Phenotype. Double mutants of *UBC4* and *UBC5* display a variety of phenotypes including slow growth and extreme sensitivity to chronic heat stress and to growth on the amino acid analogue canavanine (Seufert & Jentsch, 1990). In addition, *UBC4* single mutants show reduced turnover rates for the yeast MATα2 repressor (Chen et al., 1993) and for the noncleavable fusion protein Ub-pro-β-gal (Johnson et al., 1992).

If the ubiquitination of UBC4 facilitated any of the processes reflected by the above phenotypes, then the nonubiquitinatable mutants ubc4R64 and ubc4R144 might be expected to display some or all of these defects. We tested this possibility by introducing low-copy plasmids for *UBC4*, *ubc4R64*, and *ubc4R144* (driven by the native *UBC4* promoter) into the *ubc4ubc5* double mutant and assessing their growth and viability under two forms of environmental stress: canavanine sensitivity and chronic heat stress. It is clear from Table 1 that mutations at positions 64 and 144 have no detrimental effect on the ability of cells to survive exposure to either stress. In addition, cells carrying these mutations display characteristically normal growth rates relative to cells carrying wild-type *UBC4* (data not shown).

Table 1: Stress Phenotypes of Nonubiquitinatable ubc4 Mutants[a]

UBC4	resistance to canavanine (%)[b]	growth at 38 °C[c]
wt	69	+
R64	69	+
R144	62	+

[a] The ubc4ubc5 double mutant, MHY508, expressing wild-type UBC4 (wt) or one of the nonubiquitinatable ubc4 mutants (R64 and R144) was examined for canavanine and heat sensitivity as described in Experimental Procedures. [b] Calculated as the number of colonies present on plates containing canavanine relative to the number of colonies on plates lacking canavanine. [c] (+) and (−) indicate growth or no growth, respectively.

Table 2: Steady-State Levels of Ubiquitin X-β-Galactosidase in Nonubiquitinatable ubc4 Mutants[a]

	units of β-galactosidase activity		
UBC4 plasmid	control	Ub-pro-β-gal	Ub-met-β-gal
wt	7	300	1600
R64	9	410	1300
R144	8	520	1300

[a] β-Galactosidase activity was measured for the ubc4ubc5 double mutant, MHY508, expressing wild-type UBC4 (wt) or one of the nonubiquitinatable ubc4 mutants (R64, R144), in combination with either a control plasmid (pES12), Ub-pro-β-galactosidase (Ub-pro-β-gal), or Ub-met-β-galactosidase (Ub-met-β-gal).

In another experiment we examined the effect of the R64 and R144 mutations on the turnover of Ub-pro-β-gal by measuring the steady-state levels of Ub-pro-β-gal in *ubc4ubc5* mutant cells carrying the plasmids described above. Johnson et al. (1992) have previously shown that the metabolic instability of Ub-pro-β-gal is UBC4 dependent and results from the recognition of the Ub portion of the fusion as a degradation signal. While there appears to be slight stabilization of Ub-pro-β-gal in R64 and R144 mutants (Table 2), normal variation in β-galactosidase determinations makes it impossible to attach significance to these differences. Thus it would appear that the ubiquitination of UBC4 *in vivo* is not essential to any of the UBC4-dependent processes examined above. Although there may be other subtle phenotypes associated with UBC4 for which K144 ubiquitination is important, the significance of this result lies in its inference that UBC4 is capable of interacting with itself *in vivo*.

To prove that UBC4 does indeed interact with itself, an *in vitro* cross-linking study was done in which purified radiolabeled UBC4 was incubated in the presence of the amino group specific cross-linker BS^3 (Figure 6). The addition of the cross-linker to UBC4 resulted in the appearance of two UBC4 cross-linked species with apparent molecular masses of 36 and 47 kDa. These values are consistent with the molecular masses predicted for a UBC4 dimer (32 kDa) and a UBC4 trimer (48 kDa), respectively. The reduced intensities of these species at the higher cross-linker concentration probably reflect a reduction in the number of unmodified lysine residues that are available to complete the cross-link. The fact that the addition of a 10-fold molar excess of BSA to the UBC4 cross-linking reaction

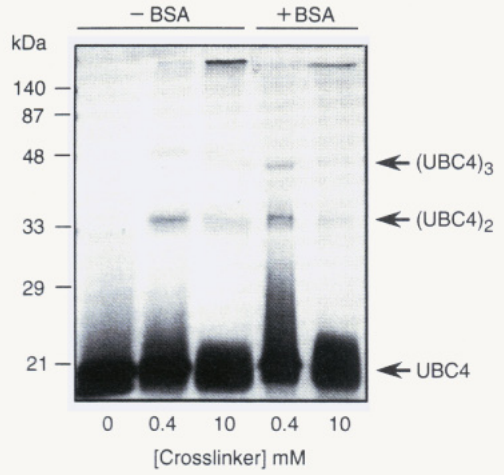

FIGURE 6: UBC4 can be cross-linked to itself. An autoradiograph of purified radiolabeled UBC4 incubated in the presence or absence of cross-linker and in the presence or absence of a 10-fold molar excess of BSA. The migrations of monomeric UBC4, dimeric $(UBC4)_2$, and trimeric $(UBC4)_3$ species are indicated relative to molecular weight standards.

(as a nonspecific negative control) neither significantly decreased the yield of these species nor resulted in the appearance of new UBC4−BSA cross-linked species indicates that the cross-linking of UBC4 to itself reflects a specific interaction between UBC4 monomers *in vitro*. The apparent reduction in the molecular weight of the UBC4 trimer in the presence of BSA is an artifact of the high BSA concentration in this region of the gel.

DISCUSSION

The present study demonstrates that the monoubiquitination of the yeast Ub conjugating enzyme UBC4 *in vivo* is a highly specific intermolecular reaction that involves a single target lysine (K144) and that depends upon the presence of active UBC4. The observation that Ub cannot be transferred from the E2 active site to the target lysine (K144) of the same molecule is easily rationalized by the distance that separates these two residues, as indicated on the three-dimensional structure of UBC4 (Figure 7) originally determined by Cook et al. (1993).

The trivial explanation that a fraction of the UBC4 expressed in yeast is either damaged or abnormal and is therefore targeted by the Ub-dependent proteolytic system for degradation can be ruled out on the basis of three observations. First, the monoubiquitination of UBC4 is clearly atypical of other substrates that are degraded in a Ub-dependent manner since it lacks the multi-Ub chain that facilitates proteolysis. Second, UBC4 displays no obvious sign of metabolic instability (unpublished observation). Finally, our observation that UBC4 ubiquitination is eliminated by substitution of a single surface lysine (K64) that is distally situated with respect to the target lysine, without apparent loss of UBC4 function, is difficult to explain using conventional notions of protein damage or abnormality.

The simplest, most straightforward explanation for the ubiquitination of UBC4 is that one monomer of UBC4 transfers Ub to the target lysine of another UBC4 monomer via a direct and specific interaction that positions the active site of one monomer close to K144 of the other monomer. This conclusion is based on our observations that the reaction is intermolecular and requires both a UBC4 monomer as an acceptor of Ub and a UBC4 monomer as a donor of Ub. It is also based on the specific interaction of UBC4 monomers that is detected *in vitro* by cross-linking analysis. If our interpretation of UBC4 ubiquitination is correct, then the necessity of positioning the active site of one monomer nearby K144 of the other monomer provides important structural information for determining the geometrical or-

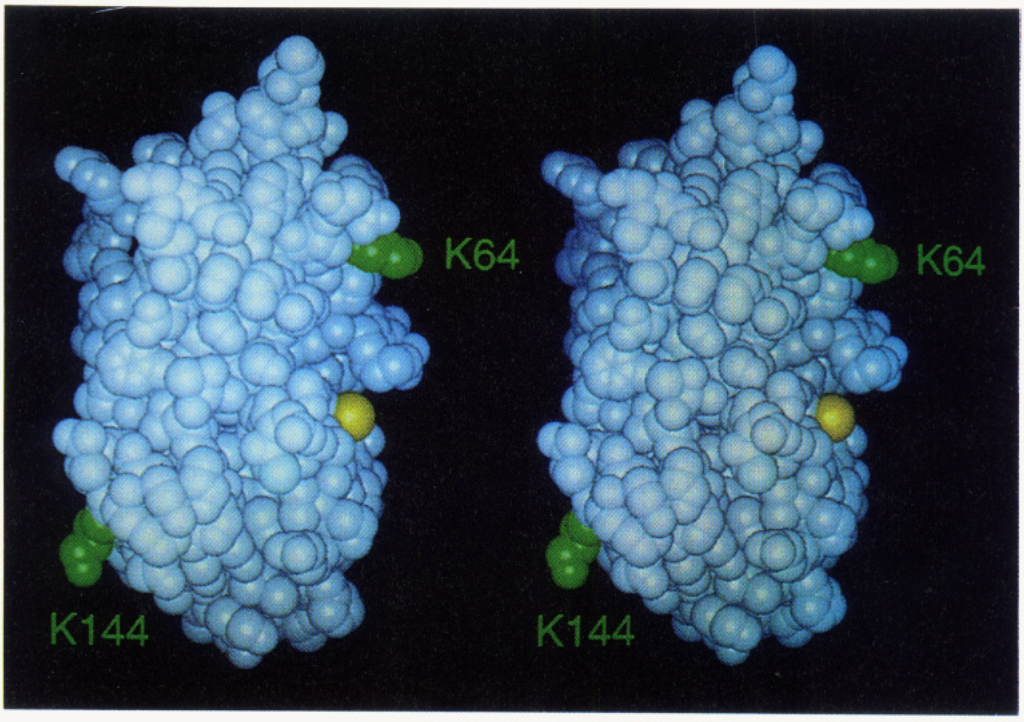

FIGURE 7: 3-D space-filling image of UBC4 showing the positions of the active site cysteine (C86), the ubiquitinated lysine (K144), and the effector lysine (K64). The image was reconstructed from the crystallographic coordinates of UBC4 using the Biosym Insight II molecular modeling package.

ganization of both Ub and UBC4 within the dimeric complex, particularly in view of the known structures for Ub (Vijay-Kumar et al., 1987), the K48-linked Ub dimer (Cook et al., 1992), and UBC4 (Cook et al., 1993).

The interaction of UBC4 with itself, which is inferred from its ubiquitination *in vivo* and which has been demonstrated directly by cross-linking analysis, strengthens the viewpoint that E2−E2 interactions constitute a general phenomenon. Other examples of such interactions include *in vitro* chromatographic evidence indicating that several E2s associate as stable homodimers or higher molecular weight multimers (Pickart & Rose, 1985; Haas & Bright, 1988: Girod & Vierstra, 1993). In addition to this *in vitro* evidence, there are other examples of E2−E2 interactions formed *in vivo*. Genetic evidence indicates that CDC34 (UBC3) is capable of interacting with itself and RAD6 (UBC2) and that these types of associations are necessary for CDC34's cell cycle function (Silver et al., 1992; Ptak et al., 1994). In recent work Chen et al. (1993) have presented evidence for the interaction of UBC7 both with itself and with UBC6 and have implicated the association of UBC6 and UBC7 in the degradation of the yeast MATα2 transcriptional regulator. Based on the degree of evolutionary conservation exhibited by all E2s identified to date, it is reasonable to expect that these E2−E2 interactions, whether they are of the homo variety or the hetero variety, will ultimately conform to a common structural theme.

It is possible that the monoubiquitination of UBC4 is facilitated by the interaction of UBC4 with another factor, possibly a ubiquitin protein ligase (E3). Although the UBC4−UBC4 interaction detected *in vitro* is specific, an inability to detect formation of the homodimer by gel exclusion chromatography (results not shown) indicates that it is also weak. This observation, coupled with the fact that K144 is not specifically targeted for ubiquitination *in vitro* using purified components (results not shown), may indicate that another protein stabilizes the UBC4−UBC4 interaction sufficiently for the transfer of Ub from one UBC4 monomer to the other to occur *in vivo* or positions K144 in a conformation that is favorable for ubiquitination. Furthermore, the observation that UBC4 does not serve as a substrate for multi-Ub chain assembly is clearly atypical of most if not all *in vivo* substrates of ubiquitination reported thus far. The fact that even free Ub functions as a substrate for the further additon of Ub (Chen & Pickart, 1990) would suggest that UBC4 is part of a complex whose subunit geometry precludes the possibility of multi-Ub chain assembly at K144. Finally, the observation that UBC4 ubiquitination is eliminated by mutation of K64, a lysine that is situated on the E2 surface far removed from the ubiquitinated position, suggests that K64 is normally involved in stabilizing the interaction of a protein with UBC4 that is necessary for UBC4 ubiquitination. At the present time we cannot distinguish whether this other protein is the UBC4 molecule that donates Ub to the UBC4 acceptor or the putative factor discussed above. If it is the putative factor, then the site-specific ubiquitination of UBC4 may prove to be an effective biochemical assay for its purification.

The function of UBC4 ubiquitination is uncertain. As pointed out above, UBC4 displays no obvious signs of metabolic instability; therefore, if UBC4 monoubiquitination fulfills an autoregulatory function, it is by a mechanism based on the modification of UBC4 and not its degradation. Furthermore, mutations of UBC4 that fail to become ubiquitinated have no adverse effect on the growth or stress-related functions of UBC4 or on the UBC4-dependent turnover of Ub-pro-β-gal. Therefore, the biological ramifications of this specific reaction remain to be addressed.

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