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A novel protein modification pathway related to the ubiquitin system

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Ubiquitin conjugation is known to target protein substrates primarily to degradation by the proteasome or via the endocytic route. Here we describe a novel protein modification pathway in yeast which mediates the conjugation of RUB1, a ubiquitin-like protein displaying 53% amino acid identity to ubiquitin. We show that RUB1 conjugation requires at least three proteins *in vivo*. ULA1 and UBA3 are related to the N- and C-terminal domains of the E1 ubiquitin-activating enzyme, respectively, and together fulfil E1-like functions for RUB1 activation. RUB1 conjugation also requires UBC12, a protein related to E2 ubiquitin-conjugating enzymes, which functions analogously to E2 enzymes in RUB1–protein conjugate formation. Conjugation of RUB1 is not essential for normal cell growth and appears to be selective for a small set of substrates. Remarkably, CDC53/cullin, a common subunit of the multifunctional SCF ubiquitin ligase, was found to be a major substrate for RUB1 conjugation. This suggests that the RUB1 conjugation pathway is functionally affiliated to the ubiquitin–proteasome system and may play a regulatory role.
Keywords: CDC53/cullin/NEDD8/RUB1/ubiquitin

Introduction

Ubiquitin is an abundant, highly conserved 76 amino acid protein found in all eukaryotic cells either free or covalently attached to cellular proteins (reviewed by Ciechanover, 1994; Jentsch and Schlenker, 1995; Hochstrasser, 1996; Varshavsky, 1997). Conjugation of ubiquitin to other proteins involves the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue of an acceptor protein. All known functions of ubiquitin are thought to be mediated through this reaction. Conjugated ubiquitin can be a substrate for further ubiquitination reactions and, indeed, most substrates appear to be modified by multiubiquitin chains in which single ubiquitin molecules are linked via isopeptide bonds. Depending on which lysine residue of ubiquitin is used for the conjugation of another ubiquitin molecule, different types of multi-ubiquitin chains can be formed *in vivo* (Hochstrasser, 1996; Varshavsky, 1997). The majority of cellular ubiquitin conjugates appear to be targeted to the 26S proteasome, which degrades the substrates to small peptides, whereas ubiquitin is recycled. Certain cell surface proteins, however, when they are modified by ubiquitination, appear to be targeted for lysosomal degradation via the endocytic route (Kölling and Hollenberg, 1994; Galan *et al.*, 1996; Hicke and Riezman, 1996; Galan and Haguenauer-Tsapis, 1997). It can therefore be assumed that ubiquitin functions primarily as a post-translationally added targeting module directing the conjugated substrates to different proteolytic systems.

Conjugation of ubiquitin proceeds via a reaction cascade involving ubiquitin-activating (E1), ubiquitin-conjugating (E2) enzymes and, at least in some cases, ubiquitin–protein ligases (E3) (Ciechanover, 1994; Jentsch and Schlenker, 1995; Scheffner *et al.*, 1995; Hochstrasser, 1996; Varshavsky, 1997). The E1 enzyme hydrolyses ATP and, via an E1-bound ubiquitinyl adenylate intermediate, forms a high energy thioester between a cysteine of its active site and the C-terminus of ubiquitin. Ubiquitin is then passed on to E2 enzymes, which form thioester-linked complexes with ubiquitin in a similar fashion. Finally, ubiquitin is covalently attached to the substrate protein by the E2 enzymes or, alternatively, by E3 enzymes which may possess substrate-binding properties (Scheffner *et al.*, 1995). In yeast, E1 is encoded by a single gene, *UBA1* (McGrath *et al.*, 1991), whereas families of E2 and E3 enzymes exist, indicating that E2 and E3 enzymes mediate the specificity of the system (Jentsch, 1992; Hochstrasser, 1996; Varshavsky, 1997)

The recent discoveries of ubiquitin-like proteins in apparently all eukaryotic cells suggest that post-translational modification of proteins by the covalent attachment of other proteins is more common than previously expected. In contrast to ubiquitin, these proteins seem to play non-proteolytic roles. Conjugation of the mammalian protein UCRP, a distant ubiquitin relative resembling two tandem copies of ubiquitin, appears to target conjugates to the cytoskeleton (Haas *et al.*, 1987; Loeb and Haas, 1992, 1994). SUMO-1, a small ubiquitin-like protein from higher eukaryotic cells, was found covalently linked to RanGAP1, the activating protein of the Ran GTPase involved in the regulation of nucleocytoplasmic trafficking (Matunis *et al.*, 1996; Johnson and Hochstrasser, 1997; Mahajan *et al.*, 1997; Saitoh *et al.*, 1997). Conjugation of SUMO-1 to RanGAP1 targets the otherwise cytosolic protein to the nuclear pore complex (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). SMT3, a yeast ubiquitin-like protein displaying 50% sequence identity to SUMO-1, is essential for viability, but its cellular function is presently unknown (Johnson *et al.*, 1997). The enzymes involved in the conjugation of SMT3 to other proteins have been identified recently. Two proteins, AOS1 and UBA2, are needed for SMT3 activation (Dohmen *et al.*, 1995; Johnson *et al.*, 1997). Interestingly, AOS1 resembles the N-terminal

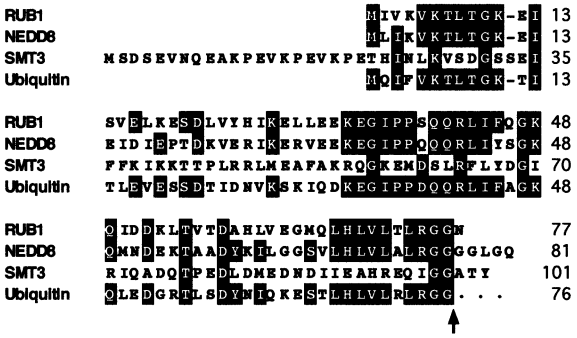

Fig. 1. Sequence comparison of RUB1 (DDBJ/EMBL/GenBank accession No. Y16890) with mouse NEDD8, yeast SMT3, and ubiquitin. Residues which are conserved between ubiquitin and the ubiquitin-like proteins are boxed. Processing sites of the precursors are indicated by an arrow.

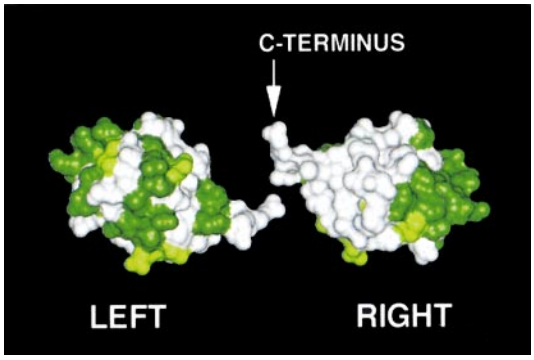

Fig. 2. Residues conserved between ubiquitin and RUB1 drawn into the known structure of ubiquitin (Vijay-Kumar *et al.*, 1987). Identical residues are shown in white, similar residues in light green, and dissimilar residues in dark green. Left and right views are shown, and the C-terminus of ubiquitin through which ubiquitin is attached to other proteins is indicated. Note that identical residues are clustered on the right side of the molecule and at a domain close to the C-terminus. The coordinates were obtained from the Brookhaven database and displayed by Insight™ software (Biosym Technology).

domain, whereas UBA2 resembles the C-terminal domain of the E1 ubiquitin-activating enzyme, UBA1. This indicates that the two proteins form a heterodimer with E1-like activity (Johnson *et al.*, 1997). Conjugation of SMT3 and SUMO-1 also involves an E2 enzyme, UBC9, which closely resembles ubiquitin-conjugating enzymes structurally (Johnson and Blobel, 1997; Saitoh *et al.*, 1998; Schwarz *et al.*, 1998).

In this study, we identify a novel ubiquitin-like protein from yeast, RUB1, which appears to be conserved from yeast to mammals. Similarly to ubiquitin, RUB1 can be conjugated to other proteins *in vivo*. We describe the mechanism by which RUB1 is conjugated and identify the enzymes required for RUB1 activation and conjugation *in vivo*. Intriguingly, CDC53/cullin, a common subunit of the SCF ubiquitin ligase, was found to be a major substrate of this novel post-translational modification system, indicating that RUB1 conjugation may regulate ubiquitin conjugation pathways.

Results

RUB1, a novel ubiquitin-like protein from yeast

In a search for new components of ubiquitin-related pathways, we identified in the yeast *Saccharomyces cerevisiae* genome a gene for a novel ubiquitin-like protein termed RUB1 (related to ubiquitin 1; Hochstrasser, 1996; Figure 1). The *RUB1* open reading frame (ORF) is interrupted by a single intron (at codon 50) and encodes a 77 residue protein which is particularly related (59% identity) to the mammalian ubiquitin-like protein NEDD8 (Kumar *et al.*, 1992), suggesting that the two proteins are functional orthologues. RUB1 displays 53% sequence identity with ubiquitin and 18 and 25% with the ubiquitin-like proteins SMT3 from yeast (Johnson *et al.*, 1997) and SUMO-1 from higher eukaryotes (Matunis *et al.*, 1996; Mahajan *et al.*, 1997), respectively. Interestingly, most residues conserved between RUB1 and ubiquitin cluster on one side of the three-dimensional structure of ubiquitin and, in particular, to a domain close to the C-terminus through which ubiquitin is conjugated to other proteins (Figure 2). This conservation led us to speculate that RUB1 may be conjugated to other proteins analogously to ubiquitin (see below).

To assess the *in vivo* function of RUB1, yeast strains lacking the *RUB1* gene were constructed. Conventional gene replacements, however, affected the expression of an adjacent gene (not shown). We therefore generated a null mutant by introducing a frameshift at codon 15 of the *RUB1* sequence. The introduction of the frameshift allele into the genome could be followed by the appearance of a novel restriction site. Haploid and diploid homozygous yeast mutants lacking functional copies of *RUB1* were viable and showed no detectable mutant phenotype (not shown). The null mutants grew at wild-type rates and displayed no obvious defects when grown under starvation or other stress conditions (e.g. heat stress, cadmium, canavanine). Thus, unlike ubiquitin (Özkaynak *et al.*, 1987; Varshavsky, 1997) or SMT3 (Johnson *et al.*, 1997), RUB1 is not essential for normal cell growth and viability.

RUB1 is conjugated to other proteins in vivo

The predicted primary translation product of RUB1 contains 77 amino acid residues and terminates C-terminally with two glycine residues followed by a single asparagine residue (Figure 1). As ubiquitin matures from precursors by proteolytic cleavage C-terminal of a similarly positioned double glycine sequence, and becomes conjugated to other proteins via the processed C-terminus (Özkaynak *et al.*, 1987; Varshavsky, 1997), we postulated that RUB1 might be subject to an analogous fate. To identify possible RUB1 conjugation reactions *in vivo*, we constructed an epitope-tagged variant of RUB1 by fusing sequences encoding a haemagglutinin (HA) epitope to the N-terminus of RUB1 (^{HA}RUB1). When this construct was expressed in wild-type yeast cells, Western blot analysis detected a protein of the predicted size of ^{HA}RUB1 (Figure 3). Notably, a limited number of additional HA-antibody-reactive proteins with sizes ranging from 30 to ~100 kDa could be identified, suggesting that these proteins represent RUB1–protein conjugates formed *in vivo* (Figure 3). Interestingly, these additional proteins could also be detected when we used a variant of ^{HA}RUB1 lacking the C-terminal asparagine residue, indicating that, analogously to ubiquitin, RUB1 matures to a protein with a C-terminal double glycine motif. The extra immunoreactive proteins were

absent when an ^{HA}RUB1 derivative was expressed which lacked the C-terminal glycine residues (Figure 3). We thus conclude that after proteolytic removal of the C-terminal asparagine residue from RUB1's precursor, the matured 76 residue RUB1 protein can be conjugated to a limited number of other proteins via its C-terminal glycine residue.

RUB1 activation is mediated by the ULA1–UBA3 E1 enzyme pair

The observation that RUB1 can be attached to other proteins *in vivo* suggested that this reaction may also involve specific activating and conjugating enzymes. Activation of ubiquitin is mediated by an ~100 kDa E1 enzyme, and the yeast enzyme, UBA1, is encoded by a gene essential for viability (McGrath *et al.*, 1991). In addition to *UBA1*, the *S.cerevisiae* genome contains several

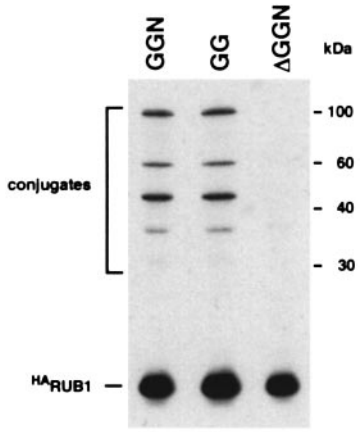

Fig. 3. Conjugate formation of RUB1 and RUB1 variants. Expression of N-terminally HA-tagged RUB1 (^{HA}RUB1) in wild-type yeast leads to the formation of a set of ^{HA}RUB1–protein conjugates (detection by Western blot). ^{HA}RUB1 without the C-terminal Asn residue (GG) is conjugated, but ^{HA}RUB1 lacking the C-terminal sequence GlyGlyAsn (ΔGGN) is not.

UBA1-related genes. Two of them, *AOS1* and *UBA2*, were shown recently to encode proteins which form a heterodimer with activating activity for the ubiquitin-like protein SMT3 (Dohmen *et al.*, 1995; Johnson *et al.*, 1997). Since no difference in RUB1 conjugation was found in either *uba1* or *uba2* temperature-sensitive mutants (not shown), we postulated that other enzymes might be needed for RUB1 activation.

Indeed, we identified two additional *UBA1*-related genes in the yeast genome which we termed *ULA1* (ubiquitin-like protein activation) and *UBA3* (ubiquitin-like protein-activating enzyme). *ULA1* encodes a protein (52.9 kDa; 462 amino acids; Figure 4A) which displays significant similarities to the N-terminal domain of UBA1 and, in particular, resembles AOS1 structurally over its entire length (Figure 4B). The other gene, *UBA3*, encodes a smaller protein (33.3 kDa; 299 amino acids; Figure 4A) which is homologous to UBA2 and to the C-terminal domain of UBA1 (Figure 3B). Notably, the UBA3 protein bears a cysteine residue at a position similar to the active site cysteine residues of UBA1 and UBA2 (within similarity box III; Figure 4) required for ubiquitin and SMT3 activation, respectively. Given these similarities, we considered the possibility that, analogous to the AOS1–UBA2 pair, ULA1 and UBA3 may combine to form an enzyme with E1-like activity for RUB1 activation, with UBA3 being the catalytically active component of the complex. To test these ideas, we generated null mutants of both genes. The *ULA1* gene was completely replaced by the yeast *LEU2* gene and a *uba3* null mutant was generated by replacing 60% of the *UBA3* ORF by the *HIS3* marker gene. The phenotypes of the null mutants subsequently were analysed after tetrad dissection of diploid heterozygous mutants. Similar to strains lacking RUB1, *ula1* and *uba3* knock-out strains are viable and exhibit no obvious growth defects (not shown).

Using these mutants, we next tested whether ULA1 and

A

ULA1

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  1 MERYDRQLRLWGALGQDSLNRSRVCVVGPATPLLQEVFKNLVLAGISSLTWLKVECAVQSGS
 63 LFLAELKKDLEPLASKQLEYEENDLRKTLQQPQYDWTRFSVVILTCIGEQTAMLDLNEIRRQ
125 RGTKFPPVLNTFVSGFYGYIYLVLSETHFVLQAHPDSKKYDLRLQNPWPELINYVDTFDLSK
187 MDTATFSGIPYTVLLMKCIAKLERDGNNGRITIDQMKKVLDQICLPLGNDVIYEPNYVEAKR
249 YAYLACSQNDCCKELEDLLRNLEISDYGNDWHDTYNYEILTLLLTLKNIAKENGELSFQPLT
311 GTLPDMESTTENYIRLKKLYEVKAKLDKSRVEESLARSKKIVSQDVLETFCSHYGEVRKILP
373 PKSDLLGIFSTSNALLDALVMVQFWEQPAVTAEDKDEFIGLRVDDNYSVMAFFGGAVVQEAI
435 KLITHHYVPIDNLFLYNGINNSSATYKI
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UBA3

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  1 MDCKILVLGAGGLGCEILKNLTMLSFVKQVHIVDIDTIELTNLNRQFLFCDKDIGKPKAQVA
 63 AQYVNTRFPQLEVVAHVQDLTTLPPSFYKDFQFIISGLDAIEPRRFINETLVKLTLESNYEI
125 CIPFIDGGTEGLKGHVKTIIPGITACWECSIDTLPSQQDTVPMCTIANNPRCIEHVVEYVST
187 IQYPDLNIESTADMEFLLEKCCERAAQFSISTEKLSTSFILGIIKSIIPSVSTTNAMVAATC
249 CTQMVKIYNDLIDLENGNNFTLINCSEGCFMYSFKFERLPDCTVCSNSNSN
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UBC12

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  1 MLKLRQLQKKKQKENENSSSIQPNLSAARIRLKRDLDSLDLPPTVTLNVITSPDSADRSQSP
 63 KLEVIVRPDEGYYNYGSINFNLDFNEVYPIEPPKVVCLKKIFHPNIDLKGNVCLNILREDWS
125 PALDLQSIITGLLFLFLEPNPNDPLNKDAAKLLCEGEKEFAEAVRLTMSGGSIEHVKYDNIV
187 SP
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B

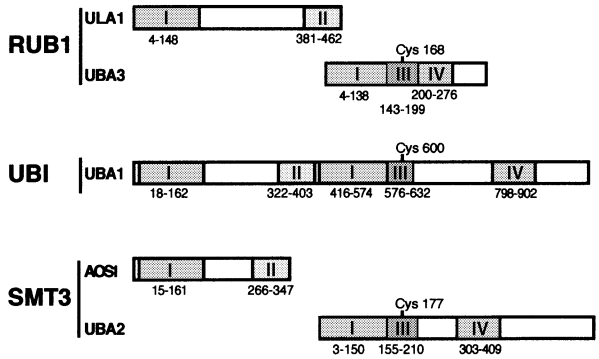

Fig. 4. (**A**) Protein sequences of ULA1, UBA3 and UBC12 (DDBJ/EMBL/GenBank accession Nos. Y16889, Y16891 and X9942, respectively). The putative active site cysteines in UBA3 and UBC12 are marked by an asterisk. (**B**) Schematic representation of the similarity domains between UBA1 and related proteins. The putative active site cysteines are located within similarity box III (termed the UBA domain), as indicated. The similarity boxes correspond to those of Johnson *et al.* (1997).

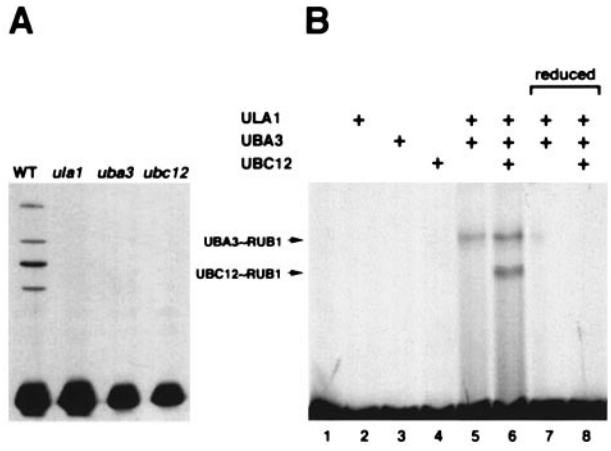

Fig. 5. The RUB1 conjugation pathway. (**A**) *In vivo* conjugation of ^{HA}RUB1 in *ula1*, *uba3* and *ubc12* null mutants is not detected, compared with wild-type. (**B**) Thioester complex formation between ^{33}P-labelled RUB1 and the enzymes of the RUB1 conjugation system. In addition to [^{33}P]RUB1 and ATP, assays contain control extracts of insect and bacterial cells (lane 1), and GST–ULA1, UBA3 and UBC12, as indicated (lanes 2–6). The bands represent thioester-linked complexes of UBA3 with RUB1 (UBA3–RUB1) and of UBC12 with RUB1 (UBC12–RUB1). Thioester formation requires the presence of ULA1. Thioester-linked complexes disappear after boiling under reducing conditions (lanes 7 and 8).

UBA3 are required for RUB1–protein conjugate formation *in vivo*. Indeed, when we expressed ^{HA}RUB1 in each of the two mutants, only ^{HA}RUB1 but no additional HA-antibody-reactive proteins could be identified (Figure 5A). We thus conclude that ^{HA}RUB1 conjugation to cellular yeast proteins depended on the presence of both ULA1 and UBA3. Furthermore, we asked whether ULA1–UBA3 functions enzymatically similarly to the E1 ubiquitin-activating enzyme by assaying for thioester formation of the recombinant proteins with radiolabelled RUB1 in the presence of ATP *in vitro*. For these assays, ULA1 was expressed as a fusion with glutathione-*S*-transferase (GST) and purified from *Escherichia coli* cells, whereas UBA3 was expressed by the baculovirus expression system in insect cells. As shown in Figure 5B, only protein mixtures containing both ULA1 and UBA3 mediated the formation of a radiolabelled complex of ~40 kDa, consistent with the size of an adduct between RUB1 and UBA3 (Figure 5B, lane 5). This complex was sensitive to boiling under reducing conditions, indicating that the proteins are indeed linked via a thioester bond (Figure 5B, lane 7). Neither ULA1 nor UBA3 alone were capable of forming a thioester complex with RUB1. This indicates that a ULA1–UBA3 pair is needed for RUB1 activation, both *in vitro* and *in vivo*.

RUB1 conjugation is mediated by the UBC12 E2 enzyme

Yeast cells possess 13 different E2 enzymes which bear a conserved UBC domain in which the active site cysteine residue is located (Jentsch *et al.*, 1990; Jentsch, 1992). One of these enzymes, UBC9, was shown recently to be specific for the ubiquitin-like protein SMT3 (Johnson and Blobel, 1997; Saitoh *et al.*, 1998; Schwarz *et al.*, 1998), but most other E2s are known to mediate ubiquitin conjugation. We investigated whether any of these E2s may function in the RUB1 conjugation pathway. When we expressed ^{HA}RUB1 in all 13 different mutant strains, no difference in RUB1 conjugation compared with wild-type was found (not shown), with the exception of *ubc12* null mutants (Figure 5A). In these strains, virtually no RUB1–protein conjugates were detected. UBC12 (21.2 kDa; 188 amino acids; Figure 4A) carries the hallmark of a typical E2, i.e. a UBC domain containing a putative active site cysteine residue for thioester formation at a conserved position. Similar to other genes of the RUB1 system, *UBC12* is not essential for viability (not shown). When we added an extract from *E.coli* cells expressing UBC12 to our thioester assays (see above), we detected, in addition to the UBA3–RUB1 thioester, a band of ~30 kDa, consistent with the size of a UBC12–RUB1 thioester complex (Figure 5B, lane 6). Complex formation was dependent on ULA1 and UBA3 in the assay and, characteristically for a thioester complex, the adduct was sensitive to boiling under reducing conditions (Figure 5B, lane 8). Thus, UBC12 functions enzymatically analogously to ubiquitin-conjugating enzymes, but is part of the RUB1 conjugation system.

CDC53/cullin is a major substrate of the RUB1 conjugation system

Although the pathway of RUB1 conjugation is remarkably similar to that of the ubiquitin system, the small number of detectable RUB1–protein conjugates (see Figure 3) is strikingly different from the numerous ubiquitin–protein conjugates present in yeast cells *in vivo* (Seufert and Jentsch, 1990). This suggests that RUB1 conjugation is highly selective and probably not involved in bulk protein degradation. To investigate the cellular role of RUB1 conjugation, we decided to identify the major substrates of this pathway. One prominent ^{HA}RUB1 conjugate (~100 kDa) was particularly abundant in nuclear fractions (not shown). We isolated this conjugate from an ^{HA}RUB1-expressing yeast culture by a procedure involving anti-HA affinity chromatography (see Materials and methods; Figure 6A). The eluted material was purified further by SDS–gel electrophoresis. The ~100 kDa protein was excised from the gel and tryptic fragments of the protein were analysed by mass spectrometry. The determined sizes of 17 fragments corresponded to predicted tryptic fragments of CDC53, a 94 kDa yeast protein required for G_1–S cell cycle progression (Mathias *et al.*, 1996; Willems *et al.*, 1996). Intriguingly, CDC53 (also known as yeast cullin) is a common subunit of the SCF ubiquitin ligase, an enzyme complex which catalyses the conjugation of ubiquitin to specific cellular substrates which are then degraded by the proteasome (see Discussion). This suggests that the RUB1 conjugation system is functionally tied to the ubiquitin–proteasome-dependent proteolytic system. To confirm the identity of the conjugate, we expressed epitope-tagged CDC53 (^{HX}CDC53) in wild-type and various null mutants of the RUB1 pathway. ^{HX}CDC53 of wild-type cells was found to run as a doublet in SDS gels, but the slower migrating protein was completely absent in *rub1*, *ula1*, *uba3* and *ubc12* null mutants (Figure 6B, and data not shown). When we expressed a larger variant of RUB1 (^{HA}RUB1) in the *rub1* null mutant, an even slower migrating antibody-reactive band appeared. Modification of ^{HX}CDC53 by ^{HA}RUB1 was verified further by immunoprecipitation and Western

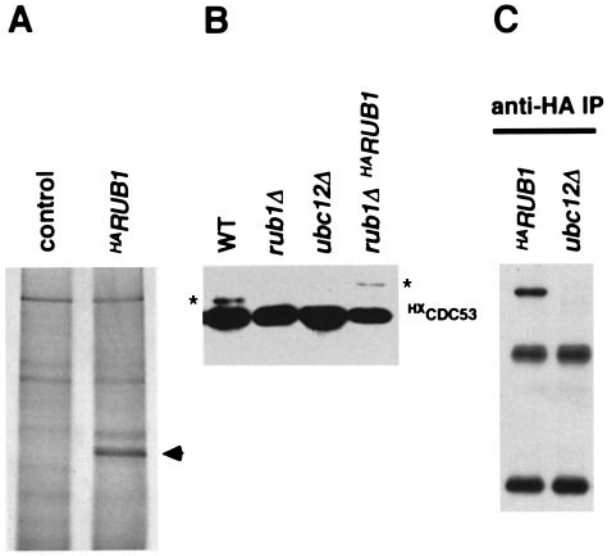

Fig. 6. Identification of CDC53 as an *in vivo* substrate of the RUB1 system. (**A**) Silver-stained gel showing an ~100 kDa conjugate purified by anti-HA–Sepharose. The protein (indicated by an arrowhead) was only present in extracts from ^{HA}RUB1-expressing cells but not in control extracts. (**B**) N-terminally tagged CDC53 (^{HX}CDC53) is modified *in vivo* by RUB1 in wild-type cells (WT) but the modification (protein bands indicated with asterisks) is absent in *rub1* and *ubc12* null mutants. In *rub1* mutants expressing ^{HA}RUB1, a larger conjugate with ^{HX}CDC53 appears. (**C**) Immunoprecipitation of ^{HA}RUB1 results in co-precipitation of Xpress™-tagged CDC53 (^{HX}CDC53, upper band), which is detectable by tag-specific antibodies (Invitrogen) in a Western blot (^{HA}RUB1). Co-precipitation of ^{HX}CDC53 is not observed in a *ubc12* null mutant (*ubc12*Δ). The lower bands correspond to heavy and light antibody chains.

blotting (Figure 6C). We thus conclude that a fraction of CDC53 is modified by a single RUB1 moiety in a reaction which requires the UBC12 E2 enzyme. This modification was resistant to reducing agents (not shown; Willems *et al.*, 1996), indicating that RUB1 is not linked via a thioester, but most likely via an isopeptide bond analogous to ubiquitin linkages. We suggest that the previous report (Willems *et al.*, 1996) that CDC53 is modified by ubiquitination was possibly mistaken as anti-ubiquitin antibodies had been employed which readily react with RUB1 (not shown).

Discussion

A novel ubiquitin-related protein

Ubiquitin is one of the most highly conserved eukaryotic proteins known to date. Human ubiquitin differs from its yeast homologue by only three amino acid residues (out of 76) and the proteins are functionally equivalent. In addition to ubiquitin, eukaryotes also express ubiquitin-related proteins, and some of these proteins can be found with conserved sequences in animals, plants and yeasts. Two classes of ubiquitin-related proteins can be distinguished. Proteins of the first class lack the double glycine motif at the C-terminal end of the ubiquitin moiety which is required for precursor processing and conjugation. Consequently, these proteins are not conjugated to other cellular proteins. The ubiquitin-related proteins of this class do not seem to promote selective proteolysis but play strikingly diverse cellular roles. The yeast ubiquitin-like protein RAD23, for instance, is implicated in DNA repair and, together with another ubiquitin-related protein, DSK2, is also involved in spindle pole body duplication (Watkins *et al.*, 1993; Biggins *et al.*, 1996). The mammalian ubiquitin-related protein BAG-1 (Takayama *et al.*, 1995), however, functions as a regulatory cofactor of the Hsc70 chaperone (Höhfeld and Jentsch, 1997; Takayama *et al.*, 1997; Zeiner *et al.*, 1997). The significance of the ubiquitin-like domain of this class of proteins remains largely enigmatic, but it has been suggested that these domains may prime the proteins for ubiquitin-dependent degradation (Varshavsky, 1997).

Proteins of the second class of ubiquitin-like proteins are distinguished by their property of becoming post-translationally attached to other cellular proteins. Known members are the interferon-inducible protein UCRP, apparently restricted to mammalian cells (Haas *et al.*, 1987; Loeb and Haas, 1992, 1994), the higher eukaryotic protein SUMO-1 (Matunis *et al.*, 1996; Johnson and Hochstrasser, 1997; Mahajan *et al.*, 1997; Saitoh *et al.*, 1997; Saitoh *et al.*, 1998), and SMT3, its apparent yeast orthologue (Johnson *et al.*, 1997). Also these proteins do not seem to be involved directly in protein degradation but appear to function as post-translationally added protein targeting devices. The yeast protein RUB1, described herein, represents a novel ubiquitin-like protein of this class. RUB1 exhibits 53% sequence identity with ubiquitin and is thus the closest homologue of ubiquitin known to date. Similarly to ubiquitin, RUB1 conjugation *in vivo* requires activating and conjugating enzymes. Intriguingly, the activating enzyme consists of two separate proteins, ULA1 and UBA3, analogous to the AOS1–UBA2 pair required for SMT3 activation. However, whether the distinct E1 subunits may also assemble to ULA1–UBA2 and AOS1–UBA3 pairs for other functions is currently speculative. The E2 enzyme required for RUB1 conjugation *in vivo*, UBC12, bears a typical UBC domain and resembles ubiquitin-conjugating enzymes over its entire length.

Mammalian NEDD8, a likely orthologue of yeast RUB1

The RUB1 system described here is most likely evolutionarily conserved. The probable mammalian orthologue of yeast RUB1 is NEDD8 (Kumar *et al.*, 1992) which displays 58% sequence identity with RUB1 (Figure 1). Similarly to RUB1, NEDD8 can be conjugated to other proteins *in vivo* (Kamitani *et al.*, 1997). Intriguingly, expression of NEDD8 in yeast results in a RUB1-like conjugate pattern that includes NEDD8-modified yeast CDC53 (D.Liakopoulos and S.Jentsch, in preparation). Moreover, the size of the major NEDD8 conjugate in mammalian cells (Kamitani *et al.*, 1997) is consistent with the predicted size of an NEDD8-modified cullin protein, suggesting that the NEDD8 pathway is functionally equivalent to the RUB1 system of yeast. The expression of NEDD8 is down-regulated during embryonic development (Kumar *et al.*, 1992). Interestingly, in adult mice, NEDD8 mRNA levels are particularly high in heart and skeletal muscle (Kamitani *et al.*, 1997). These tissues are characterized by a high protein turnover rate and a very limited capacity to divide. Thus, RUB1/NEDD8 pathways may be relevant specifically for cells which have exited the cell cycle (see below).

Cullin as a substrate

Although our current work has not defined the cellular function of the RUB1 conjugation system, the data presented indicate that this pathway is affiliated with the ubiquitin system. Surprisingly, our studies identified CDC53 as a major substrate of this novel modification system. CDC53, a member of the evolutionarily conserved family of cullin proteins (Kipreos *et al.*, 1996), is a common subunit of the SCF ubiquitin ligase which ubiquitinates a variety of substrates promoting their destruction (Mathias *et al.*, 1996; Feldman *et al.*, 1997; Skowyra *et al.*, 1997). SCF complexes also contain SKP1 and alternative substrate-specific F-box proteins. It has been shown that the degradation of the cyclin kinase inhibitor SIC1 is mediated by the UBC3 (CDC34) ubiquitin-conjugating enzyme and SCF^{Cdc4}, containing CDC53, SKP1 and the F-box protein CDC4 (Schwob *et al.*, 1994; Mathias *et al.*, 1996; Feldman *et al.*, 1997; Skowyra *et al.*, 1997). Conversely, CLN1-cyclin degradation is thought to be mediated by UBC3 and a similar complex, SCF^{Grr1}, which, however, contains the alternative F-box protein GRR1 (Barral *et al.*, 1995; Willems *et al.*, 1996; Li and Johnston, 1997; Skowyra *et al.*, 1997). We observed no influence on the overall stability of CDC53 in *rub1* mutants (not shown), suggesting that the modification of CDC53 by RUB1 does not seem to target the protein for proteasomal degradation. It is thus conceivable that, analogously to SUMO-1, RUB1 may play a non-proteolytic role. We speculate that the modification of CDC53 by RUB1 conjugation might, for example, influence SCF's subunit composition or activity, directing the complex towards specific SCF substrates. In fact, we observed a genetic interaction (synthetic lethality) of *rub1*, *ula1*, *uba3* and *ubc12* null mutants with *ubc3*/*cdc34* temperature-sensitive alleles (not shown), indicating that the RUB1 modification pathway is linked to the function of this cell-division cycle protein. Fluorescence-activated cell sorter (FACS) analysis of *rub1* null mutants, however, has not revealed any significant cell-cycle progression defect (not shown). This suggests that RUB1-modified SCF, possibly involving F-box proteins other than CDC4 or GRR1, may act specifically on substrates whose stabilization in *rub1* null mutants is not deleterious for cell growth or division. Experiments designed to identify proteins which specifically associate with the RUB1-modified form CDC53 (SCF) are currently underway and are expected to reveal insights with respect to the cellular role of this novel protein modification pathway.

Materials and methods

Cloning and yeast techniques

The yeast techniques used here are described in Ausubel *et al.* (1994). All strains are derivatives of DF5 (Finley *et al.*, 1985; *ura3-52 leu2-3, -112 lys2-801 trp1-101 his3*Δ*200*). The *RUB1* gene, including its intron and 220 bp upstream and 360 bp downstream sequences, was cloned into pUC19 (Ausubel *et al.*, 1994) via PCR, creating plasmid pUCz48. A *rub1-1* null mutant was created by replacing the *Bgl*II fragment of *RUB1* by a double-stranded oligonucleotide, which introduces a frameshift at codon 15. This manipulation results in the appearance of a new stop at codon 16 and also in the introduction of an *Xba*I site. The mutant allele was cloned into YIplac211 (Gietz and Sugino, 1988), and the resulting plasmid YIrub1, which contains the *URA3* marker, was used to generate a *rub1-1* null strain (YDM1, *MAT***a** *rub1-1*). Replacement of *RUB1* wild-type by the *rub1-1* allele was performed by the two-step gene replacement technique (Ausubel *et al.*, 1994) as follows: the plasmid YIrub1 was linearized at the unique *Bsa*BI site within *rub1-1* and transformed into DF5 diploids. These subsequently were sporulated and, after tetrad dissection, ura^+ haploids were cultured and plated onto 5-fluoro-orotic acid (5-FOA) plates to select for eviction of the *URA3* marker. 5-FOA-resistant haploids were then tested for retention of the *rub1-1* allele by PCR amplification of the *RUB1* locus and digestion of the product with *Xba*I. Positive clones were finally tested by Southern blotting.

UBC12, including its intron, was cloned from a λEMBL3A library using gene-specific probes and subcloned into pBluescript (Stratagene), generating pKM050. A *ubc12*Δ deletion construct (pKM051) was made by replacing the promoter region and 70% of the ORF with the *TRP1* gene and was used for constructing a *ubc12*Δ strain (YDM3, *MAT***a** *ubc12::TRP1*).

ULA1 was cloned from genomic sequences by PCR and cloned into pGEX2TK (pGEX-ULA); *UBA3* was cloned by PCR into pBluescript (pBS-UBA3) and pVL1392 (pVL-UBA3). A *ula1*Δ strain (YGD1, *MAT***a** *ula1::LEU2*) was made by replacing the entire ORF by the *LEU2* gene; a *uba3*Δ strain (YGD4, *MAT***a** *uba3::HIS3*) was made by replacing 60% of the coding region, including the sequence encoding the putative active site, by the *HIS3* gene. For thioester experiments, the *RUB1*-coding sequence ending at codon 76 was cloned into pGEX2TK, generating pGEXRUB. Genes encoding N-terminally triple HA-tagged versions of RUB1 were placed under the control of the *ADH1* promoter in YIplac204 (Gietz and Sugino, 1988). Plasmid KB622 was the source for epitope-tagged CDC53 (gift of Daniel Kornitzer). The gene for CDC53, tagged with both His_6 and an Xpress™ tag (Invitrogen) (^{HX}CDC53), was cloned into YIplac211 under the control of the *GAL1-10* promoter. Genes for HA-tagged versions of RUB1 were integrated into the *TRP1* locus and for tagged CDC53 into the *URA3* locus.

Protein techniques

For Western blots, yeast protein extracts were prepared by boiling cells in Laemmli buffer. Standard techniques for SDS–PAGE and Western blotting were used (Ausubel *et al.*, 1994).

The RUB1 conjugate was isolated as follows: yeast spheroplasts from a 9 l YPD culture of a haploid strain expressing HA-tagged RUB1 (see above) were suspended in 18% Ficoll 400, 20 mM KPO_4 pH 6.45, 5 mM $MgCl_2$ and lysed in a Dounce homogenizer (Hurt *et al.*, 1988). The lysate was centrifuged at 13 000 *g*, and the pellet containing organelles and intact nuclei was resuspended in 50 mM Tris–HCl pH 7.5, 1% Triton X-100, and applied to a DEAE column (Pharmacia). Bound proteins were eluted with an NaCl gradient (0–300 mM). Fractions containing RUB1 conjugates were then pooled, concentrated, incubated with 1 ml of 12CA5 antibody-coupled protein A–Sepharose and eluted by competition with 1 mg of HA peptide. The eluate was concentrated and proteins were visualized by Coomassie Brilliant Blue staining after SDS–PAGE (Figure 5A). A prominent band at ~100 kDa was excised from the gel and subjected to MALDI mass spectrometry (Mortz *et al.*, 1994).

Thioester assays

For thioester assays (Scheffner *et al.*, 1993), RUB1 was obtained by purifying a GST–RUB1 fusion [containing a thrombin cleavage and a protein kinase A (PKA) phosphorylation site] from *E.coli* cells expressing pGEXRUB using a glutathione–Sepharose column. The fusion protein was ^{33}P-labelled by PKA, cleaved with thrombin, thrombin was heat-inactivated, and radiolabelled RUB1 was used for thioester assays. In these assays, extracts of insect cells expressing UBA3, *E.coli* extracts containing UBC12, and purified GST–ULA1 were used and incubated in 25 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM ATP and 0.1 mM dithiothreitol. The reaction products were analysed by non-reducing SDS–PAGE followed by autoradiography.

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