A family of structurally related RING finger proteins interacts specifically with the ubiquitin-conjugating enzyme UbcM4[1]

Gustavo Martinez-Noel[a], Rainer Niedenthal[b], Teruko Tamura[b], Klaus Harbers[a,*]

[a] *Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistr.52, D-20251 Hamburg, Germany*
[b] *Institut für Physiologische Chemie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30623 Hannover, Germany*

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Abstract The ubiquitin-conjugating enzyme UbcM4 was previously shown to be necessary for normal mouse development. As a first step in identifying target proteins or proteins involved in the specificity of UbcM4-mediated ubiquitylation, we have isolated seven cDNAs encoding proteins that specifically interact with UbcM4 but with none of the other Ubcs tested. This interaction was observed in yeast as well as in mammalian cells. With one exception, all UbcM4-interacting proteins (UIPs) belong to a family of proteins that contain a RING finger motif. As they are structurally related to RING finger proteins that have recently been shown to play an essential role in protein ubiquitylation and degradation, the possibility is discussed that UIPs are involved in the specific recognition of substrate proteins of UbcM4.

Key words: Ubiquitin-conjugating enzyme; RING finger protein; Protein-protein interaction

1. Introduction

Modification of proteins by the attachment of the polypeptide ubiquitin is of major importance for a variety of cellular processes. In most cases this modification serves as a signal for selective degradation of ubiquitylated proteins by the proteasome (for review see [1–3]). The enzymatic machinery that covalently links ubiquitin to target proteins is highly conserved in eukaryotes. In an initial step the ubiquitin-activating enzyme (E1 or Uba) forms a thioester bond with the C-terminus of ubiquitin, which is then transferred to a specific cysteine residue of an ubiquitin-activating enzyme (E2 or Ubc). Although E2 enzymes can directly donate ubiquitin to proteins, ubiquitin protein ligases (E3 or Ubr) are typically required for the specific recognition of substrates and, at least in one case, have been shown to be directly involved in the final transfer of ubiquitin to a protein [4]. E2 enzymes are encoded by a multigene family and show extensive sequence homology among each other, suggesting a high degree of functional redundancy. However, results from mutational analysis suggest that individual E2 enzymes are involved in the ubiquitylation of specific target proteins and that their function can not be replaced by other members of the E2 family (e.g. [5]).

We recently described a transgenic mouse mutant where partial inactivation of the gene for the ubiquitin-conjugating enzyme UbcM4 resulted in a recessive-lethal phenotype [6]. Homozygous embryos die in utero most likely as a result of placenta impairment. As a first step in identifying possible target proteins of UbcM4 or proteins that are involved in the specificity of UbcM4-mediated ubiquitylation, we here describe the isolation and characterization of proteins that can physically interact with UbcM4.

2. Materials and methods

2.1. Yeast two-hybrid screening

The yeast two-hybrid screen was performed essentially as described [7]. Yeast strain L40, containing the bait plasmid pLexA/UbcM4 encoding the LexA protein fused in frame to the complete coding region of the mouse UbcM4 cDNA [8], was transformed with a day 10.5 mouse embryo cDNA expression library fused in frame to the transcription activation domain of plasmid pVP16. Transformants were selected for growth on plates lacking histidine and supplemented with aminotriazole. DNA was prepared from all positive clones and used to transform $CaCl_2$-competent cells of the bacterial strain HB101 (leuB auxotroph). Bacterial clones containing only the cDNA plasmids were isolated by selection on M9(−Leu) plates. After isolation from bacterial cultures all cDNA plasmids were retested individually for interaction with UbcM4 by using the yeast YRN974 strain containing as a reporter gene the green fluorescent protein (GFP) as described previously [9].

Constructs containing the UbcH1/HHR6B [10], UbcM3 [11], UbcH5 [12], UbcH8 [13] or mUbc9 [14] cDNAs fused in frame to the LexA binding domain were obtained by PCR amplification of the complete coding regions of the respective cDNAs followed by ligation into vector pBTM116.

2.2. Cloning of UbcM4-interacting protein (UIP) cDNAs

To obtain the cDNA clones pUIP28 and pUIP48, which contain the complete protein coding region for UIP28 and UIP48, respectively, total liver RNA from BALB/c mice was reverse transcribed, as previously described [15], and amplified by PCR using primers derived from the sequence of the UIP28 homologous rat gene RBCK1 [16] or UIP48 homologous EST clones. The amplified fragments were directly cloned into the *Eco*RV site of the Bluescript vector. The nucleotide sequences of pUIP28 and pUIP48 have been submitted to the GenBank/EMBL Data Bank with accession numbers AF124663 and AF124664, respectively.

2.3. In vivo binding assays with tagged proteins

UbcM4 tagged with 6×His at its C-terminus was obtained by PCR amplification of the complete protein coding region of the UbcM4 cDNA [8] and cloning of the amplified fragment into the *Bam*HI and *Eco*RI sites of plasmid CMVc-Jun-His [17] from which the c-Jun insert had been excised. The plasmids pUIP28-HA, pUIP48-HA and pKIAA-HA containing the hemagglutinin (HA) tag with the sequence YPYDVPDYA at the C-terminus of the full-length protein were obtained by standard PCR techniques using as templates the plasmids pUIP28, pUIP48 and pKIAA0161 (a gift from Dr. T. Na-

*Corresponding author. Fax: (49) (40) 48051188.
E-mail: harbers@hpi.uni-hamburg.de

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Abbreviations: E1/Uba, ubiquitin-activating enzyme; E2/Ubc, ubiquitin-conjugating enzyme; E3/Ubr, ubiquitin-protein ligase; EST, expressed sequence tag; GFP, green fluorescent protein; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; UIP, UbcM4-interacting protein

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gase, Kazusa DNA Research Institute, Kisarazu, Japan). The amplified fragments were cloned in the *Eco*RI site of vector pHCMV-G [18] from which the insert was removed. All constructs were sequenced in order to confirm their structure.

Human 293 kidney cells were plated at a density of 3×10^6 on 90 mm diameter dishes 20 h before transfection. After 16 h the medium was changed. The cells were transfected by the calcium phosphate precipitation method [19] with 20 μg of the respective plasmid. Transfection efficiencies were controlled by transfecting in parallel a plate with the plasmid pCMVlacZ and measuring the β-galactosidase activity. The precipitate was removed after 4 h and fresh medium was added. After 48 h protein extracts were prepared by resuspending the cells in 1.5 ml lysis buffer (20 mM Tris-HCl pH 8.0, 1% NP40, 0.1% SDS, 5 mM imidazole, 15 0mM NaCl, protease inhibitor cocktail [Complete, EDTA-free, Boehringer]) and sonication. Cell debris was removed by centrifugation and the supernatant added to 100 μl of a 50% slurry of TALON resin (Clontech). After 30 min at room temperature the resin was pelleted by centrifugation and washed twice with 1 ml lysis buffer, twice with 1 ml wash buffer containing 20 mM Tris-HCl (pH 8.0), 0.1% NP40, 500 mM NaCl, and once with 1 ml of the same wash buffer containing no NaCl. The bound proteins were eluted with a buffer containing 20 mM Tris-HCl pH 8.0, 0.1% NP40, 150 mM imidazole. After precipitation with acetone overnight at −20°C the proteins were analyzed by SDS-PAGE (12% gel) and Western blotting. The HA-tagged protein was detected using the mouse monoclonal antibody 12CA5 (Boehringer Mannheim) and for detection of (His_6)-tagged proteins BMG-His-1 (Boehringer) was used as the primary antibody. Primary antibodies were visualized by the Immun-Star chemiluminescent assay kit (Bio-Rad). For protein extraction under denaturing conditions cells were suspended in a lysis buffer containing 80 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS and 100 mM dithiothreitol.

2.4. Sequence comparison

Multiple alignments of conserved sequence motifs were constructed by using the DNASIS program (Hitachi) and by further adjustments made by hand. In some cases the PSI-BLAST search program [20] was used. Conserved amino acid residues were classified according to the following physico-chemical properties: weakly hydrophilic (N, Q, S, T), acidic (D, E), basic (H, K, R), small (A, G), hydrophobic (I, L, M, V), and aromatic (F, W, Y). Coiled coil domains were predicted by using the COILS method [21].

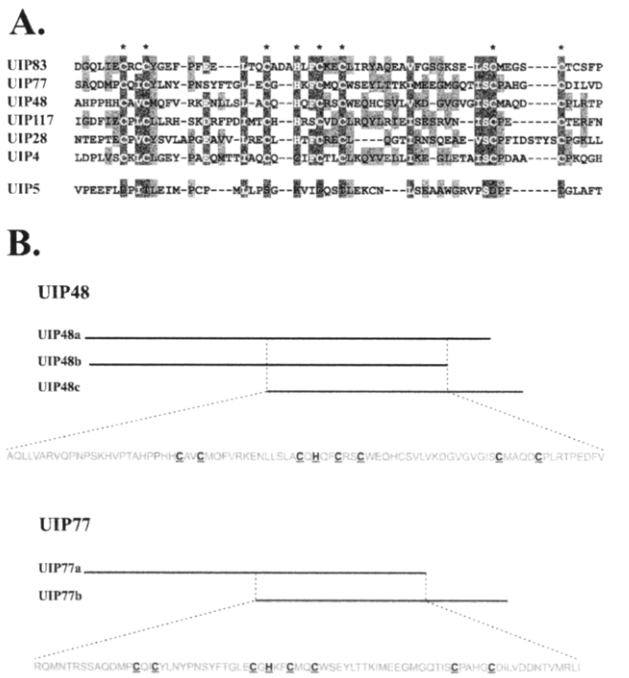

Fig. 1. RING finger motifs of UIPs. A: Sequence alignment of RING finger motifs present in UIPs. The conserved cysteine and histidine residues of the the RING finger motif are marked by asterisks. White letters on dark background indicate residues that are conserved in at least 80% and dark letters on gray background indicate residues that are conserved in 50–80% of the aligned sequences. Black letters on dark background are residues that replace the conserved cysteine and histidine residues. For details of sequence alignments see Section 2. B: Schematic representation of partial cDNA clones encoding UIP48 and UIP77, respectively. The amino acid sequence of the overlapping region is shown and conserved cysteine and histidine residues of the RING finger motif are underlined. Amino acids are given in the single-letter code.

3. Results

3.1. Isolation and characterization of UIPs

When a cDNA expression library from mouse embryos was screened with a bait expression vector containing the LexA binding domain fused to the complete coding region of UbcM4, seven different cDNAs were identified that specifically interacted with UbcM4. The proteins encoded by these cDNA clones will be referred to in this report as UIPs (UbcM4-interacting proteins). As a further control for the specificity of interaction the following ubiquitin-conjugating enzymes were tested: UbcH1/HHR6B [10], UbcM2 [11], UbcM3 [11], UbcH5 [12], UbcH8 [13] and mUbc9 [14] – a conjugating enzyme for the ubiquitin-related protein SUMO-1 [22,23]. No interaction was seen between these E2 enzymes and the UIPs when GFP was used as a reporter gene in the yeast two-hybrid screen (data not shown). The only exception might be UIP48, which showed some interaction with UbcH8 and UbcM3.

Sequence analysis showed that, with the exception of UIP5, all UIP clones contained a RING finger motif (Fig. 1A). The RING finger constitutes a cysteine-rich region which can be described as Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$X_{(4-48)}$-Cys-X_2-Cys, where X can be any amino acid, although there are clear preferences for particular residues at certain positions [24]. The RING motifs found in the UIPs are in agreement with this consensus sequence, the only exception being UIP4 where the His is replaced by Cys. UIP5 contains a sequence motif that has several amino acids in common with the RING motif of the other UIPs, however, the cysteine and histidine residues are replaced by other residues (Fig. 1A). The region containing the RING finger also seems to be responsible for the interaction with the UbcM4 protein, as in two cases partial cDNA clones coding for the same protein were found to overlap only in the sequence encoding the RING motif (Fig. 1B).

Comparison of amino acid sequences predicted from the open reading frames of the UIP clones with protein sequences of the GenBank data base revealed that UIP28 is 97% identical with the rat RBCK1 protein [16], UIP48 is 100% identical with the mouse Ariadne-2 protein (accession number AJ130975), UIP4 is 95% identical with the product of the human gene KIAA0161 (accession number D79983), UIP5 is 84% identical with the human KIAA0860 protein (accession number AB020667), UIP117 is 59% identical with a mosquito blood meal-induced protein (accession number U84248) and UIP77 is 55% identical (73% similar) with the protein encoded by the *Drosophila* gene Ariadne-1 (accessiom number X98310). No significant homology was found for UIP83. Partial sequences and the overall structure of the five UIPs or UIP-homologous proteins for which the complete amino acid sequence is known are shown in Fig. 2. All five proteins pos-

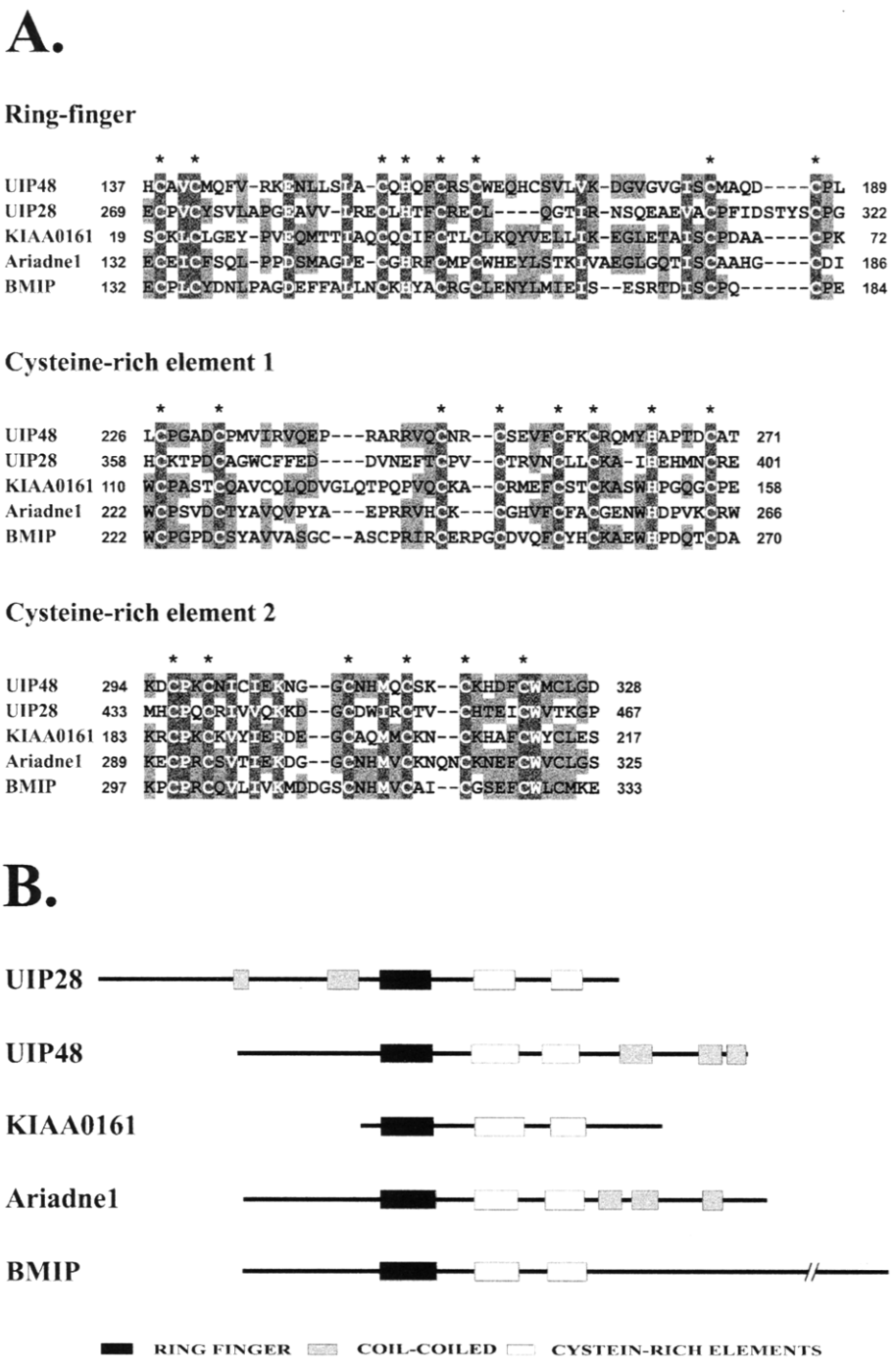

Fig. 2. Cysteine/histidine-rich motifs present in UIPs and UIP-homologous proteins. A: Amino acid sequence alignment of RING finger, Cys-rich region 1 and Cys-rich region 2 of UIP48, UIP28, KIAA0161 (human homologue of UIP4), Ariadne-1 (*Drosophila* homologue of UIP77), and mosquito blood meal-induced protein (BMIP; mosquito homologue of UIP117). Conserved cysteine and histidine residues, which may form zinc fingers, are marked by asterisks. White letters on dark background indicate residues that are conserved in 100% of the aligned sequences and black letters on gray background those that are conserved in 75% of the aligned sequences. The numbers at the left and right indicate amino acid positions at the termini of the aligned blocks. These numbers are based on the sequences deposited into the GenBank data base under the following accession numbers: AF124663 (UIP28), AF124664 (UIP48), D79983 (KIAA0161), X98310 (Ariadne-1), and U84248 (BMIP). B: Schematic drawing of the structural arrangement of UIPs and UIP-homologous proteins showing the positions of the different sequence motifs discussed in Section 3. For details of the sequence alignment see Section 2.

sess three Cys-rich regions, namely a RING finger, and two other potential zinc binding domains referred to in Fig. 2B as Cys-rich region 1 and Cys-rich region 2. These two domains are related to the previously described B-box and B-box-like motifs [25]. In addition, three proteins (UIP28, UIP48 and Ariadne-1) are predicted to have two coiled-coil domains. Proteins containing RING finger, B-box and coiled-coil domains are designated the RBCC protein family, which includes several transcription factors and proto-oncogenes [24]. In summary, at least five of the initially isolated seven UIPs are structurally closely related and at least three are members of the RBCC family of proteins.

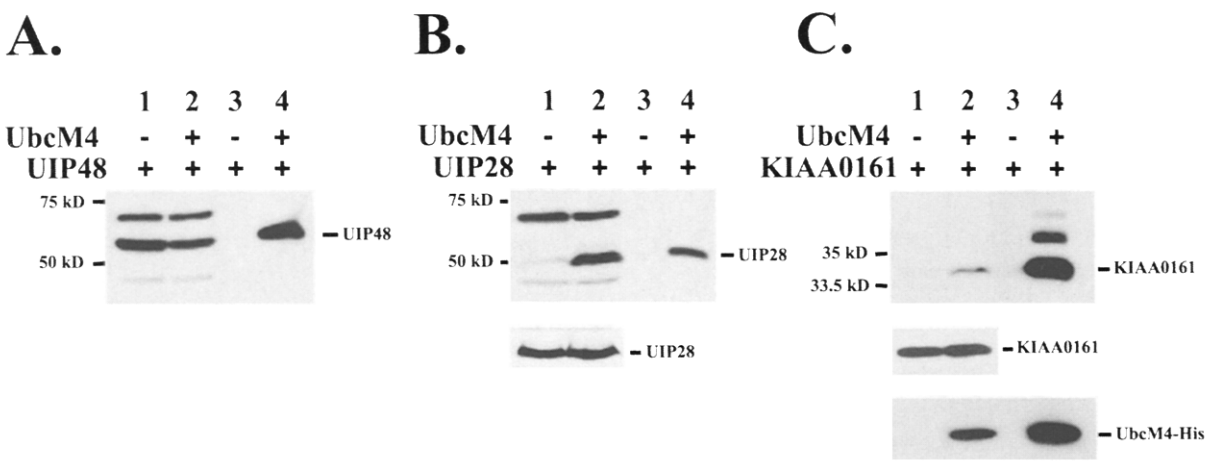

Fig. 3. Association of UbcM4 with UIP48, UIP28 and KIAA0161 in mammalian cells. A: Cell extracts were prepared by standard NP40 lysis from 293 cells transfected with HA-tagged UIP48 alone (lanes 1 and 3) or together with His_6-tagged UbcM4 (lanes 2 and 4). Proteins were analyzed directly (lanes 1 and 2) or after metal affinity chromatography (lanes 3 and 4) by SDS-PAGE and Western blotting using a monoclonal antibody directed against the HA peptide. The upper band of about 65 kDa is unspecific and was also detected by the HA antibody in protein extracts from untransfected 293 cells (not shown). B, upper panel: Same as described for A, but HA-tagged UIP28 was used instead of UIP48; lower panel: cell extracts were prepared by SDS lysis from cells transfected with HA-tagged UIP28 alone (lane 1) or together with His_6-tagged UbcM4 (lane 2) and equal amounts of protein were directly analyzed by SDS-PAGE and hybridization with the same HA antibody as described above. C, upper panel: Same as described for A, but HA-tagged KIAA0161 was used instead of UIP48; middle panel: extracts were prepared by SDS lysis from cells transfected with HA-tagged KIAA0161 (lane 1) or together with His_6-tagged UbcM4 (lane 2) and equal amounts of protein were directly analyzed by SDS-PAGE and hybridization with the HA antibody; lower panel: the blot shown in upper panel was stripped and rehybridized with a monoclonal antibody directed against the 6×His peptide. Transfection efficiencies were about the same in all experiments shown here. Therefore, identical amounts of protein from total cell extracts were applied to the gel, allowing direct comparison of band intensities between lanes 1 and 2.

3.2. Protein-protein interaction in mammalian cells

To determine whether any of the UIPs interacts with UbcM4 in mammalian cells, hexahistidine (His_6)-tagged UbcM4 and hemagglutinin (HA)-tagged UIPs were introduced into human 293 kidney cells by transient transfection of cytomegalovirus (CMV)-based expression vectors. Forty-eight hours after co-transfection the cells were lysed and the His-tagged UbcM4 purified from the protein extract by metal affinity chromatography. The proteins bound to the metal resin were separated by SDS-PAGE, transferred to a nitrocellulose filter and analyzed for the presence of UIPs by immunostaining with HA-antibodies (Fig. 3). The experiments were performed with UIP28, UIP48 and KIAA0161, the human homologue of UIP4. The main result shown in Fig. 3 is the observation that all three UIPs co-elute with the metal resin-bound UbcM4 (lane 4). In the absence of His_6-tagged UbcM4 none of the UIPs is bound to the resin (lane 3). These results clearly indicate that the UIPs can associate with UbcM4 in mammalian cells.

For comparison, total extracts of cells transfected with UIP alone or together with UbcM4 were analyzed by Western blotting and immunostaining with HA antibody (lanes 1 and 2 in Fig. 3). In the case of UIP48 the amount of the HA-tagged protein extracted by standard NP40 lysis from transfected 293 cells was about the same in the absence or presence of UbcM4 (Fig. 3A). Surprisingly, however, in the case of UIP28 and KIAA0161 the amount of the HA-tagged proteins were significantly increased when UbcM4 was co-expressed (Fig. 3B,C). These differences cannot result from ubiquitylation as the same results were obtained with a catalytically inactive UbcM4 expression plasmid where the Cys in position 86 was mutated to Ser (data not shown). However, when total proteins were extracted by lysis with SDS these differences were not observed (lower panels in Fig. 3B,C), clearly indicating that they are not due to different expression levels. At the present time we have no explanation for these results. Of particular interest is the observation that the stability of the UIPs is obviously not effected by cotransfection with UbcM4 as the steady-state level of the UIPs in total extracts is the same in the absence and presence of UbcM4.

4. Discussion

Using the yeast two-hybrid system, we have identified seven proteins, designated UIPs, that interact specifically with the E2 enzyme UbcM4. Other E2 enzymes, including UbcH8, which is closely related to UbcM4 at the amino acid sequence level, do not associate with the UIPs. The interaction is not restricted to yeast but was also observed in mammalian cells, at least for those three UIPs tested in this report. All UIPs with the exception of UIP5 contain a potential zinc binding domain generally referred to as RING finger motif. The region containing the RING motif also seems to be responsible for the interaction with UbcM4. This was demonstrated in two cases where partial cDNAs for the same UIP were isolated that interacted with UbcM4 and overlapped only in the RING finger region (see Fig. 1B).

RING finger containing proteins have been associated with a variety of different functions such as transcriptional regulation, signal transduction, oncogenesis and development (for a review see [24]). At present, the molecular function of the RING finger is unclear. Based on several observations it has been suggested that the RING motif participates directly in protein-protein interaction (e.g. [26]). Five of the UIPs, for which there is sufficient sequence information available, contain other zinc finger-like domains known as B-box motifs and in at least three cases a potential α-helical coiled-coil domain adjacent to the RING finger (Fig. 2). These last three

proteins (UIP28, UIP48, and UIP77) belong to a subgroup of RING finger proteins that is often referred to as RING-B-box-coiled coil (RBCC) proteins [24]. Several members of the RBCC subfamily have been implicated in cellular transformation when found at the breakpoint of chromosomal translocations (e.g. [27]). Although very little is known about the function of the UIPs described in this report, the fact that their amino acid sequences have been highly conserved during evolution suggests that they have important functions common to all eukaryotic organisms. UIP28 is the mouse homologue of the previously described protein RBCK1 from rat. This protein was identified by the yeast two-hybrid screen as a protein kinase C (PKC)-interacting protein and it has been speculated that it might be a transcription factor that has a role in the signaling pathway through PKC [16,28]. The genes encoding UIP77 and UIP48 are the mouse homologues of the *Drosophila* genes Ariadne-1 and Ariadne-2, respectively. In *Drosophila*, mutational analysis of these genes revealed that they are required at multiple steps during development, most notably in nervous system connectivity and oogenesis (M. Aguilera and A. Ferrus, personal communication). Nothing is known about the possible function of the other UIPs.

The possible role of the UIPs in the UbcM4-mediated ubiquitylation and degradation pathway is currently unknown. The observation that the steady-state level of the UIPs is not reduced after co-transfection with UbcM4 suggests that they themselves might not be targets of the UbcM4-mediated ubiquitylation/degradation pathway in vivo. However, further experiments to determine the half-live and ubiquitylation of UIPs are needed to conclusively answer this question. Alternatively, UIPs might represent E3s or parts of E3 complexes and, therefore, be directly involved in the recognition of target proteins of UbcM4. We currently favor the hypothesis that, in analogy to the recently identified RING finger protein Rbx1 [29,30], UIPs are involved in recruiting UbcM4 to SCF-like E3 complexes and, therefore play an essential role in UbcM4-mediated protein ubiquitylation and degradation. Indirect support for this function comes from the multi-domain structure of the UIPs, which similar to Rbx1 might allow physical interaction with several other proteins. Further evidence for the involvement of RING finger proteins in selective ubiquitylation and proteolysis comes from the RING finger proteins Sina and its human homologues. Sina is involved in the selective proteolysis of a transcriptional repressor called Tramtrack (TTK), which is required for proper eye development in *Drosophila* [31]. Similarly, human homologues of Sina (termed Siahs) are involved in regulating the proteolysis of DCC (deleted in colorectal cancer), a putative transmembrane receptor for the axon and and cell guidance factor netrin-1 [32]. Sina, as well as its human homologues, forms part of a protein complex and it has been shown that the RING finger domain is required for its interaction with ubiquitin-conjugating enzymes and hence for its function in proteolysis [33].

Based on our previous observation that inactivation of UbcM4 in mice is most likely responsible for abnormal placenta development [6], we speculate that the UIPs, in analogy to Sina, link the ubiquitin/proteasome system to proteins involved in placenta development. Experiments are under way to confirm our hypothesis by isolating and characterizing other proteins that interact with the UIPs and by eventually identifying in vivo target proteins of UbcM4.

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