

Identification of two proteins, S14 and UIP1, that interact with UCH37

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Abstract By the use of the yeast two-hybrid screen we have identified two proteins that interacted with UCH37: S14, which is a subunit of PA700 and a novel protein, UIP1 (UCH37 interacting protein 1). The interaction of UCH37 with S14 or UIP1 was confirmed by *in vitro* binding assay and *in vivo* co-immunoprecipitation analysis. The C-terminal extension of UCH37 is essential for interaction with S14 or UIP1 as shown by the yeast two-hybrid assay and the *in vitro* binding assay. Furthermore, UIP1 blocked the interaction between UCH37 and S14 *in vitro*. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Ubiquitin C-terminal hydrolase; Isopeptidase; Ubiquitin; Yeast two-hybrid system

1. Introduction

In eukaryotes, ubiquitin-dependent proteolysis is essential for selective protein degradation by the 26S proteasome. The ubiquitin-26S proteasome pathway is involved in many important cellular processes such as cell cycle regulation, differentiation, antigen presentation and the stress response etc. (reviewed in [1–3]).

The 26S proteasome is the proteolytic complex that degrades polyubiquitinated proteins [4]. It consists of two sub-complexes: the barrel-shaped proteolytic core complex (the 20S proteasome) and the 19S regulatory complex (also known as PA700). The proteolytic activity of the 20S proteasome is highly regulated by the PA700 [5]. Recently, Cohen and co-workers reported a PA700-associated isopeptidase that could selectively disassemble polyubiquitin chains from the distal end of poorly ubiquitinated protein conjugates [6,7]. This iso-

peptidase is thought to be involved in ‘editing’ ubiquitinated substrates according to the length of polyubiquitin chains rather than the structure of the target proteins themselves. The nucleotide and amino acid sequences of human ubiquitin C-terminal hydrolase (UCH)37 were deposited in GenBank by Cohen and co-workers in 1999 as the PA700-associated isopeptidase. UCH37 is a UCH that has a C-terminal extension of ~100 amino acids in length in addition to the N-terminal UCH domain.

We have identified a 26S proteasome-associated UCH (Uch2p) in fission yeast which contains a C-terminal extension in addition to its N-terminal UCH domain [8]. Uch2p is homologous (37% identical) to human UCH37 both at the N-terminal UCH domain and at the C-terminal extension. Recently the homologue of UCH37 in fruit fly was identified as a subunit of the 19S regulatory complex [9]. However, the biological functions and interacting proteins of this 19S regulatory complex-associated UCH remain unclear.

Here, we report the identification of two proteins, S14 and UCH37 interacting protein 1 (UIP1), which interact with UCH37. The proteins were identified by the use of the yeast two-hybrid screen.

2. Materials and methods

2.1. Plasmid construction

To construct a bait plasmid, UCH37/pAS2-1 (pU1), for the two-hybrid screen, a full-length open reading frame (ORF) of human UCH37 cDNA was amplified from a HeLa cDNA library and inserted into a yeast vector, pAS2-1, which expresses GAL4 DNA binding domain (DNA-BD) fused to target protein (Clontech). Two truncated UCH37 cDNAs were also inserted separately into pAS2-1 for further characterisation of positive candidates isolated from the yeast two-hybrid screen: UCH37ΔC/pAS2-1 (pU2), in which the C-terminal extension of UCH37 was deleted, and UCH37ΔN/pAS2-1 (pU3), in which the N-terminal UCH domain of UCH37 was deleted. The schematic diagram of the above plasmids constructed is shown in Fig. 1A. To generate glutathione-S-transferase (GST)-fused UCH37 and UCH37ΔC, the cDNAs of the entire ORF of UCH37 and truncated (without the C-terminal extension sequence) UCH37 were inserted into pGEX-KG (Pharmacia) to generate two plasmids: UCH37/pGEX-KG and UCH37ΔC/pGEX-KG. The entire ORF of human UCH-L3 was also inserted into pGEX-KG to generate a plasmid, UCH-L3/pGEX-KG. The cDNA of S14, obtained from the yeast two-hybrid screen, was also inserted into pGEX-KG. The 5′ primer for the S14 cDNA, incorporating a FLAG-tag sequence, was used to generate the FLAG-S14/pGEX-KG. To generate 6×His-tagged UIP1 or 6×His-tagged NonO, the cDNA of UIP1 or NonO obtained from the yeast two-hybrid screen was inserted into pQE30 (Qiagen). For mammalian expression, the cDNA of UIP1 was inserted into pcDNA3 (Invitrogen). The 5′ primer for the UIP1 cDNA, incorporating a FLAG-tag sequence, was used to generate the FLAG-UIP1/pcDNA3. The full-length ORF of UCH37 and truncated (without the C-terminal extension) UCH37 cDNAs were in-

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Abbreviations: UCH, ubiquitin C-terminal hydrolase; UIP1, UCH37 interacting protein 1; GST, glutathione-S-transferase; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HRP, horseradish peroxidase

serted into pCI-neo (Promega). The 5' primer for the UCH37 and UCH37ΔC cDNAs, incorporating a Myc-tag sequence, was used to generate Myc-UCH37/pCI-neo and Myc-UCH37ΔC/pCI-neo. All constructs were verified by DNA sequencing.

2.2. Yeast two-hybrid system

The yeast two-hybrid screen was performed using the MATCH-MAKER two-hybrid system (Clontech) according to the manufacturer's instructions. The UCH37/pAS2-1 (pU1) plasmid was transformed into yeast strain PJ69-2A, and the transformed PJ69-2A cells were mated with a pre-transformed HeLa cell cDNA library (Clontech) in yeast strain Y187. Library plasmids from positive yeast clones were subjected to DNA sequencing. β-Galactosidase activity in each of the transformants was measured according to the method of Miller [10].

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of UIP1 gene

1 ng of the first strand cDNA of multiple human tissues (Clontech) was used as template for RT-PCR. Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a control.

2.4. Expression and purification of GST fusion and 6×His-tagged proteins

Expression of the GST, GST-UCH37, GST-UCH37ΔC, GST-UCH-L3 or GST-FLAG-S14 fusion protein was induced in *Escherichia coli* strain DH5α which had been transformed with pGEX-KG, UCH37/pGEX-KG, UCH37ΔC/pGEX-KG, UCH-L3/pGEX-KG or FLAG-S14/pGEX-KG plasmid. Induction was by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. Proteins extracted from *E. coli* were purified using glutathione-agarose beads (Sigma) and analysed by SDS-PAGE followed by staining with Coomassie brilliant blue [11]. 6×His-tagged UIP1 or 6×His-tagged NonO was expressed in *E. coli* strain M15 which had been transformed with UIP1/pQE30 or NonO/pQE30. The recombinant proteins were affinity purified using Talon nickel resin (Clontech) [12].

2.5. Removal of GST-tag from GST-FLAG-S14

FLAG-S14 was generated by thrombin-mediated cleavage of GST-FLAG-S14. The procedure for this cleavage is essentially as described [11].

2.6. In vitro binding assay

Approximately 10 μg of GST protein or the fusion derivatives immobilised on glutathione-agarose beads was incubated with 10 μg of purified FLAG-S14 or 6×His-UIP1 for 2 h at 4°C in binding buffer (150 mM NaCl, 10 mM Tris pH 8.0, 0.3% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). The mixtures were then subjected to centrifugation and the pellets were washed three times with the binding buffer. The bound proteins were separated by 10% SDS-PAGE, electro-transferred onto nitrocellulose membranes and probed with either M2 monoclonal anti-FLAG antibody (Sigma) or monoclonal anti-6×His antibody (Qiagen).

2.7. In vitro competitive binding assay

Approximately 10 μg of GST-UCH37 immobilised on glutathione-agarose beads was incubated with 10 μg of FLAG-S14 or 6×His-UIP1 for 2 h at 4°C in binding buffer. Then a various amount of the third protein (as indicated in Section 3) was added to the above mixtures and incubated for another 2 h at 4°C in binding buffer. The proteins bound to the glutathione-agarose beads were centrifuged, washed and analysed as described above. 10 μg 6×His-NonO was used as a control as indicated in Section 3.

2.8. Co-immunoprecipitation

COS-1 cells were cultured in a high glucose (4500 mg/l) version of Dulbecco's modified Eagle's media (DMEM) supplemented with 10% foetal calf serum. The procedures for transfection and co-immunoprecipitation analysis are essentially as described [13].

2.9. Immunoblotting

Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The blots were probed with specific antibodies as indicated above. Goat anti-mouse polyclonal antibody

horseradish peroxidase (HRP) conjugate was used as secondary antibody. The membranes were visualised by enhanced chemiluminescence (SuperSignal West Pico, Pierce).

3. Results

3.1. Isolation of proteins that interact with UCH37 in a yeast two-hybrid screen

The yeast two-hybrid screen [14] was used to identify cellular proteins that interact with UCH37. A screen of 3×10^6 pre-transformed HeLa cell cDNA library transformants yielded 28 positive colonies that were able to grow in media without histidine and adenine, and were able to express β-galactosidase. The plasmids from eight of the 28 positive colonies had S14 cDNA inserts. The S14 protein is a subunit of the 26S proteasome [15]. The eight cDNA inserts are identical and encode the partial sequence of S14 (from amino acid number 43 to 257 of the total 257 amino acids of S14) as indicated in Fig. 1B.

The plasmids from four of the 28 positive colonies contained cDNA inserts derived from a novel gene with identical length (~1.3 kb) encoding a 358 amino acid protein as shown in Fig. 1B. We name this novel gene UIP1. The nucleotide and amino acid sequences of UIP1 were deposited in GenBank/EMBL under accession number AF267739. The deduced amino acid sequence of UIP1 is identical to an expressed-Xq28STS protein (accession number U82695, deposited in 2000). This indicates that the UIP1 gene resides on the Xq28 region of the X chromosome. Computer database searches revealed that UIP1 is a novel gene that shows

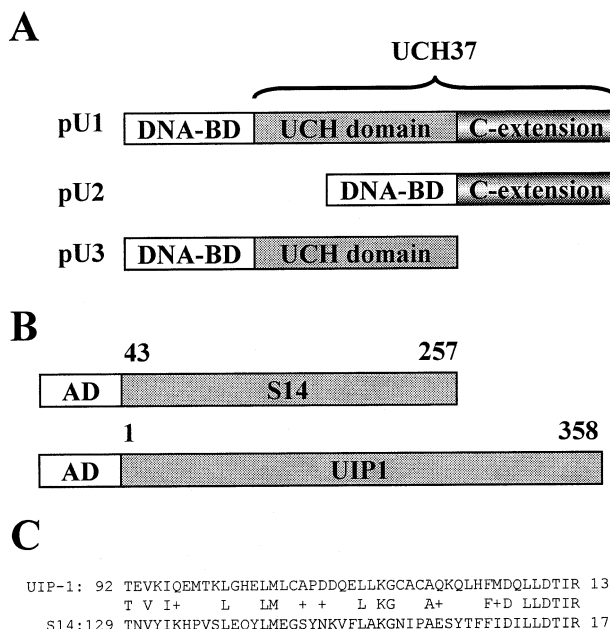


Fig. 1. A: The schematic diagram of plasmids constructed for the yeast two-hybrid assay. The full-length ORF of mouse UCH37 cDNA, a truncated UCH37 cDNA (the UCH domain is deleted) or another truncated UCH37 cDNA (the C-terminal extension was deleted) was inserted into a pAS2-1 vector to generate DNA-BD fusion plasmids: pU1, pU2 and pU3 respectively. B: The schematic diagram of hybrid AD fusion proteins obtained from the yeast two-hybrid screen. C: The homologous motif common to S14 and UIP1. DNA-BD represents the DNA binding domain and AD represents the activation domain.

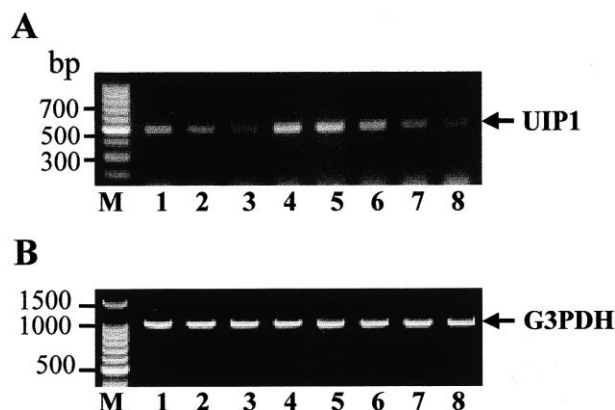


Fig. 2. The transcripts of UIP1 from multiple human tissues. A: RT-PCR was carried out to study the expression of UIP1 gene using primers specific for the UIP1 gene. Lanes 1–8 show PCR products amplified from the following tissues: 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, peripheral blood leukocyte. B: RT-PCR was carried out to amplify human G3PDH gene as a control. DNA size markers are shown on the left.

no significant homology to any known gene. Interestingly, a short stretch of amino acid sequence in UIP1 exhibits 38% identity to S14 (Fig. 1C). The plasmids of 14 of 28 positive colonies contained cDNA inserts encoding NonO, a transcriptional regulator [16].

We transformed the S14 or UIP1 plasmid isolated from the yeast two-hybrid screen individually into yeast strain Y187. The Y187 yeast strain harbouring S14/pGAD-GH or UIP1/pGAD-GH was mated with PJ69-2A strain transformed with pU1, pU2 or pU3 respectively to test the interaction between S14 or UIP1 with UCH37, UCH37 Δ C or UCH37 Δ N specifically. The β -galactosidase activity of each of the mating diploid strains was measured and several controls were also set up. As shown in Table 1, S14 was found to interact with UCH37, but was not able to interact with the N-terminal UCH domain or C-terminal extension of UCH37. The results of the yeast two-hybrid assay indicates that S14 may interact with UCH37 and that the interaction may be dependent on both the N-terminal UCH domain and the C-terminal extension of UCH37. When a similar yeast two-hybrid experiment was performed using UIP1 and UCH37, UIP1 was found to interact with UCH37 and its C-terminal extension. However,

Table 1
 β -Galactosidase activity of each of mating diploid cells harbouring DNA BD and AD fused proteins

DNA BD	AD	Relative activity ^a
p53	SV40-L-T	1.00
p53	S14	0.02
p53	UIP1	0.01
UCH37	SV40-L-T	0.03
UCH37	S14	0.18
UCH37	UIP1	0.42
UCH37 Δ C	SV40-L-T	0.01
UCH37 Δ C	S14	0.03
UCH37 Δ C	UIP1	0.33
UCH37 Δ N	SV40-L-T	0.01
UCH37 Δ N	S14	0.02
UCH37 Δ N	UIP1	0.02

^aRelative activity was calculated from the specific activity of β -galactosidase using Miller's method [10]. The values were calculated from triplicate samples for each of the diploid cells.

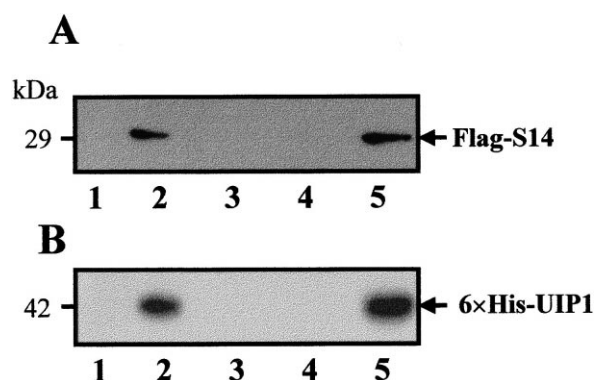


Fig. 3. In vitro binding assay. Purified FLAG-S14 (A) or 6 \times His-UIP1 was incubated with GST (lane 1), GST-UCH37 (lane 2), GST-UCH37 Δ C (lane 3) or GST-UCH37 Δ N (lane 4) bound glutathione-agarose beads. The bound proteins were washed three times, separated by SDS-PAGE, electro-transferred onto nitrocellulose membranes and probed with anti-FLAG antibody (A) or anti-6 \times His antibody (B). The purified FLAG-S14 (A) or 6 \times His-UIP1 (B) was used as a positive control (lane 5). Molecular mass markers are shown in kDa on the left.

UIP1 did not interact with the N-terminal part of UCH37 (the part of UCH37 that includes the UCH catalytic domain). We suggest that UIP1 may bind the C-terminal extension of UCH37. Furthermore, it appears that UCH37 binds UIP1 more strongly compared to its binding of S14 as judged by the β -galactosidase activity.

3.2. Transcripts of the UIP1 gene are present in multiple human tissues

The transcription of the UIP1 gene in human tissues was studied by RT-PCR. As shown in Fig. 2, bands with various brightness of the expected size (~ 500 bp) were amplified from multiple human tissues. This indicates that the UIP1 gene is expressed in many tissues with varied steady state amounts of

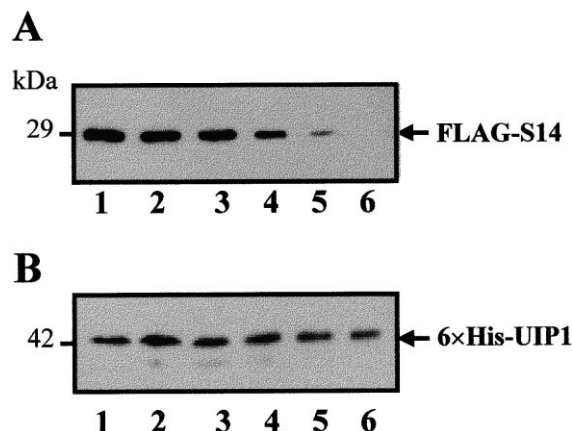


Fig. 4. In vitro competitive binding assay. A: Immobilised GST-UCH37 was incubated with FLAG-S14 first, and then 10 μ g of 6 \times His-NonO (lane 1) or the following amount of 6 \times His-UIP1 was added: 0 μ g (lane 2), 2 μ g (lane 3), 4 μ g (lane 4), 8 μ g (lane 5) or 16 μ g (lane 6). B: Immobilised GST-UCH37 was incubated with 6 \times His-UIP1 first, and then 10 μ g of 6 \times His-NonO (lane 1) or the following amount of FLAG-S14 was added: 0 μ g (lane 2), 10 μ g (lane 3), 20 μ g (lane 4), 30 μ g (lane 5) or 40 μ g (lane 6). The bound proteins were washed three times, separated by SDS-PAGE, electro-transferred onto nitrocellulose membranes and probed with anti-FLAG antibody (A) or anti-6 \times His antibody (B).

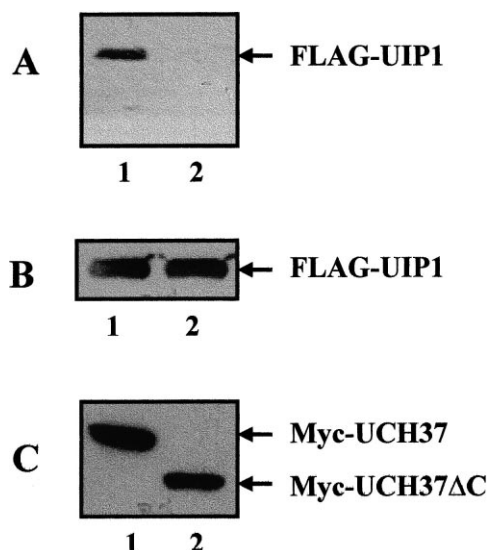


Fig. 5. Co-immunoprecipitation of Myc-UCH37 with Flag-UIP1. Cell lysates from COS-1 cells co-transfected with Flag-UIP1/pcDNA3 and Myc-UCH37/pCI-neo (lane 1) or Flag-UIP1/pcDNA3 and UCH37ΔC/pCI-neo-Myc (lane 2) were immunoprecipitated using anti-Myc antibody. The immunoprecipitates were probed with anti-Flag antibody (A) to detect co-immunoprecipitated protein(s). The expression of various constructs in both samples was verified by Western blotting of a quarter of the total cell lysates prior to co-immunoprecipitation using either anti-Flag antibody (B) or anti-Myc antibody (C).

mRNA. Bands of ~1000 bp with approximately equal intensity were amplified from multiple tissues when G3PDH was used as a control.

3.3. S14 and UIP1 bind to UCH37 in vitro

We carried out in vitro binding assays to confirm that S14 or UIP1 could individually bind UCH37 in vitro under conditions other than that of the yeast two-hybrid system. As shown in Fig. 3A,B, both the FLAG-S14 and 6×His-UIP1 bound GST-UCH37 in vitro, but the binding was not evident when the C-terminal extension of UCH37 was deleted. No binding was detected between FLAG-S14 or 6×His-UIP1 with GST or GST-UCH-L3 respectively. These results indicate that S14 and UIP1 could bind UCH37 specifically in vitro in a C-terminal extension dependent manner.

3.4. UIP1 blocks the interaction between S14 and UCH37 in vitro

As the results of experiments using the yeast two-hybrid assay showed that the interaction between UIP1 and UCH37 was much stronger than that between S14 and UCH37, it was therefore of interest to determine whether the binding of S14 by UCH37 is affected by the presence of UIP1 in vitro. As shown in Fig. 4A, the interaction of FLAG-S14 with GST-UCH37 was inhibited by 6×His-UIP1 but not by 6×His-NonO. Furthermore, the interaction of FLAG-S14 with GST-UCH37 was inhibited by 6×His-UIP1 in a concentration-dependent manner. Almost no detectable FLAG-S14 was found bound to GST-UCH37 when the molar ratio of 6×His-UIP1:FLAG-S14 was 1:1 (lane 6 of Fig. 4A). However, FLAG-S14 (even at the 6×His-UIP1:FLAG-S14 molar ratio of 1:6) had no significant effect on the amount of 6×His-UIP1 bound to GST-UCH37 (lane 6 of Fig. 4B).

This indicates that UCH37 binds UIP1 preferentially and that the binding of S14 by UCH37 may be blocked by UIP1.

3.5. Co-immunoprecipitation analysis

When using anti-Myc antibody to precipitate the lysates from COS-1 cells co-transfected with FLAG-UIP1/pcDNA3 and Myc-UCH37/pCI-neo or Myc-UCH37ΔC/pCI-neo, the FLAG-UIP1 was found to co-precipitate with Myc-UCH37, but not with Myc-UCH37ΔC (Fig. 5A). In addition, the co-immunoprecipitation of FLAG-UIP1 with Myc-UCH37 was quantitative and specific (Fig. 5B,C). This finding suggests that the UIP1 could bind UCH37 via its C-terminal extension in vivo.

4. Discussion

As the PA700-associated isopeptidase (UCH37) has been shown to edit polyubiquitinated substrates [6,7], it may play important roles in regulating protein turnover by the 26S proteasome. We have shown that the gene (*uch2⁺*) encoding the homologue (Uch2p) of UCH37 in fission yeast is not essential for cell growth [8]. However, whether UCH37 affects mammalian cell growth is still unclear. Identification of mammalian proteins that interact with UCH37 would go some way towards the elucidation of the function of UCH37. By performing the yeast two-hybrid screen using UCH37 as a bait, we have isolated a few candidates that may interact with UCH37. One candidate, S14, is a subunit of PA700 [15]. The interaction of UCH37 with S14 was further confirmed by an in vitro binding assay. The finding of the interaction between S14 and UCH37 may provide insights into the subunit arrangement of PA700, in particular the subunits of PA700 that associate with UCH37.

We did not do the co-immunoprecipitation analysis of S14 and UCH37. This is because both S14 and UCH37 are subunits of PA700, and if one subunit is precipitated, then the whole complex may be co-precipitated. Thus a positive result from a co-immunoprecipitation analysis of S14 and UCH37 does not necessarily mean that there is a direct interaction between them.

Another candidate is a transcriptional regulator, NonO [16]. However, the interaction of UCH37 with NonO was not detectable by the in vitro binding assay (data not shown). Whether UCH37 and NonO interact in vivo remains to be elucidated. Interestingly, a recently identified protein, BAP1 which contains a UCH domain, binds BRCA1 (a transcriptional regulator) RING finger domain and enhances BRCA1-mediated cell growth suppression [17]. The C-terminal extension of UCH37 exhibits extensive homology with the C-terminal tail of BAP1. It is of interest to know whether UCH37 is also involved in transcriptional regulation.

A novel protein, UIP1, was also isolated by the yeast two-hybrid screen. The interaction of UCH37 with UIP1 was confirmed by an in vitro binding assay (Fig. 4) and in vivo co-immunoprecipitation analysis (Fig. 5). The expression of the UIP1 gene was detected by Northern blotting originally. However, the signals for UIP1 transcripts in the Northern blot were very weak using either radioactive or non-radioactive labelling method. So RT-PCR was carried out to detect the transcripts of the UIP1 gene. The mRNA of the UIP1 gene was found in many tissues with varied expression level. The transcripts of the mouse homologue (75% identity) of

UIP1 gene were detected in mouse liver and testis by Northern blotting. The expression pattern of the mouse UIP1 gene is similar to that of mouse UCH37 gene (manuscript in preparation). This indicates that UIP1 and UCH37 may be functionally related.

UIP1 possesses a motif sharing high homology with S14. It is of interest to know whether this motif binds UCH37. This is being actively investigated in our laboratory. The interaction of UIP1 with UCH37 is stronger than that of UCH37 with S14 as shown by the results of the yeast two-hybrid assay and the in vitro competitive binding assay. This suggests that UIP1 may modulate UCH37's interaction with S14 in the 26S proteasome. The C-terminal extension of UCH37 confers on the enzyme distinctive features not found in other UCHs, such as UCH-L1 and UCH-L3 that possess only the UCH domains but do not have C-terminal extensions. We have shown that the C-terminal extension of Uch2p mediates the association of Uch2p (the homologue of UCH37) with the 26S proteasome in *Schizosaccharomyces pombe* [8]. In the present study we found that UCH37 interact with UIP1 and S14 in a C-terminal extension dependent manner, and UIP1 was found to interact with the C-terminal extension of UCH37 alone by yeast two-hybrid system. We postulate that UIP1 binds the C-terminal extension of UCH37 preferentially and thus blocks the association of UCH37 with PA700 in the 26S proteasome.

The biological functions of UCH37 are still unclear. Cohen and co-workers suggested that one of the major functions of this isopeptidase may be to rescue poorly ubiquitinated proteins from degradation by disassembling their short polyubiquitin chains [6,7]. However, our results from the *uch2* null mutant fission yeast strain showed that high molecular weight ubiquitin conjugates accumulated after the disruption of the *uch2*⁺ gene (manuscript in preparation). This indicates that Uch2p may facilitate the turnover of ubiquitinated proteins by the 26S proteasome, probably by trimming the ubiquitinated proteins so that they can be transported through the narrow orifice and enter the chamber of the 20S proteasome where proteolysis occurs. The truncated *uch2ΔC* (without the C-terminal extension nucleotide sequence) strain showed a phenotype similar to that of the *uch2* null mutant strain (manuscript in preparation). This indicates that the Uch2p may lose its function if disassociated from the 26S proteasome, even though the N-terminal UCH domain still pos-

sesses the UCH enzymatic function. UIP1 may modulate the ubiquitinated protein turnover by blocking the association of UCH37 with the 26S proteasome. This hypothesis warrants further investigation.

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