Characterization of *C. elegans* RING finger protein 1, a binding partner of ubiquitin-conjugating enzyme 1

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Abstract

In a yeast two-hybrid screen, RING finger protein 1 (RFP-1) and UBR1 were identified as potential binding partners of *C. elegans* UBC-1, a ubiquitin-conjugating enzyme with a high degree of identity to *S. cerevisiae* UBC2/RAD6. The interaction of RFP-1 and UBC-1 was confirmed by co-immunoprecipitation experiments. Yeast interaction trap experiments mapped the region of interaction to the basic N-terminal 313 residues of RFP-1. The acidic carboxy-terminal extension of UBC-1 was not required for the interaction with RFP-1. Western blot analysis and indirect immunohistochemical staining show that RFP-1 is present in embryos, larvae, and adults, where it is found in intestinal, nerve ring, pharyngeal, gonadal, and oocyte cell nuclei. Double-stranded RNA interference experiments against *rfp-1* indicate that this gene is required for L1 development, vulval development, and for egg laying. By contrast, RNA interference against *ubc-1* gave no obvious phenotype, suggesting that *ubc-1* is nonessential or is functionally redundant.

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Keywords: *C. elegans*; E2; *ubc-1*; RAD6; Ubiquitylation; RING; Vulva; RNA interference

Introduction

Rapid and selective degradation of cellular proteins is accomplished by the ubiquitin proteasome pathway. Ubiquitin is a highly conserved 76-residue protein that is conjugated to other proteins, marking them for degradation by the 26 S proteasome. In an ATP-dependent step, a ubiquitin-activating enzyme (E1) forms a thiol ester with the carboxyl group of glycine 76 of ubiquitin, thus activating ubiquitin’s C-terminus for nucleophilic attack. Ubiquitin is transferred to a cysteinyl residue in a ubiquitin-conjugating enzyme (UBC or E2), forming a thiol ester bond. A ubiquitin-protein ligase (E3) recognizes certain degradation signals in the substrate protein and promotes the transfer of ubiquitin from the E2 to the substrate. An isopeptide bond is formed between the carboxyl group of ubiquitin’s glycine 76 and the ε-amino group of a lysyl residue in the target protein. An important feature of ubiquitylation as a degradation signal is the conjugation of a multiubiquitin chain to a target protein. This is accomplished by successive rounds of ubiquitylation on lysine 48 of the previously conjugated ubiquitin moiety (Chau et al., 1989). Once a substrate becomes multiubiquitylated, it is recognized by a multi-subunit ATP-dependent protease called the 26S proteasome (reviewed in Jentsch and Schlenker, 1995). The target protein is cleaved into small peptides, and free ubiquitin is released by ubiquitin isopeptidases (reviewed by Pickart, 2001). Perturbations in the ubiquitin proteasome system have been associated with cancers (Aberle et al., 1997; Joazeiro et al., 1999; Orford et al., 1997; Scheffner et al., 1993; Waterman et al., 1999), Liddle’s syndrome (Abriel et al., 1999; Goulet et al., 1998; Staub et al., 1997), Parkinson’s disease (Shimura et al., 2001; Zhang et al., 2000), and Angelman syndrome (Jiang et al., 1998; Matsuura et al., 1997).

The *C. elegans* genome encodes 20 ubiquitin-conjugating enzymes and 3 ubiquitin E2 variants (uev); the latter lack an active site cysteinyl residue and probably do not conjugate ubiquitin via thiolester bonds (Jones et al., 2001). *C. elegans ubc-1* encodes a 192 amino acid protein (21.5 kDa) that is the functional homologue of the *S.
cerevisiae ubiquitin-conjugating enzyme encoded by RAD6/UBC2 (Jentsch et al., 1987; Leggett et al., 1995). RAD6 has important roles in DNA repair (Bailly et al., 1997; Hoege et al., 2002; Lawrence, 1994; Ulrich and Jentsch, 2000), histone ubiquitylation and gene silencing (Dover et al., 2002; Huang et al., 1997; Robzyk et al., 2002; Sun and Allis, 2002; Turner et al., 2002), protein degradation by the N-end rule pathway (Bachmair and Varshavsky, 1989; Bachmair et al., 1986; Dohmen et al., 1991), and spermatogenesis in mammals (Roest et al., 1991). and spermatogenesis in mammals (Roest et al., 1991).

ubc-1 was able to complement the DNA repair functions of rad6Δ yeast, although it failed to rescue the sporulation defect of these cells. UBC-1 contains the conserved UBC domain with the active site cysteinyl residue (C88) as well as a 40 residue acidic carboxy-terminal extension or tail. In vitro ubiquitin-conjugation assays showed that UBC-1 could conjugate ubiquitin via a thiolester bond (Leggett et al., 1995). Chemical cross-linking studies showed that UBC-1 formed homodimers and homotetramers. Deletion of the acidic C-terminal tail significantly reduced, but did not abolish, this self-association. The C-terminal tail may be important for specifying protein targets or modulating interactions with other proteins. In vitro assays showed that UBC-1 was conjugated to ubiquitin by the canonical thiolester bond as well as via a peptide bond. The residue that became monoubiquitylated via a peptide bond was mapped to lysine-162 in the C-terminal tail (Leggett and Candido, 1997).

The cellular roles and protein targets of UBC-1, the Rad6p homologue in C. elegans, are unknown. Herein, we use C. elegans to study RAD6 in the context of a multicellular organism. In an effort to elucidate UBC-1’s biochemical functions, a yeast two-hybrid screen was performed to identify proteins with which it interacts. Upon confirmation of the interaction between UBC-1 and its partner RING finger protein 1 (RFP-1), RNA interference and immunohistochemical staining experiments were employed to learn more about this novel interaction.

Materials and methods

A yeast two-hybrid screen for proteins that interact with UBC-1

The ubc-1 coding sequence in pBSIKS (pR1.7a) was PCR amplified with oligonucleotides EC1 (gga att cca tAT GAC GAC GCC CAG CAG TAG ACG) and EC2 (aag gag ccA AGC ATT CGA TCC ACT GGC TCC), and subcloned into the Ndel (5′) and BamHI (3′) sites of pRSETA. This clone was digested with AccI and BsmI, and the ubc-1 PCR product was replaced with ubc-1 obtained from pR1.7a. ubc-1 was cut out of pRSETA and cloned into the Ndel and BamHI sites of pAS1-CYH2. The resulting bait plasmid, pAS1-CYH2::UBC-1, contained TRP1 and the 2-μ origin for selection and replication in yeast.

General yeast protocols (Guthrie and Fink, 1991) and high efficiency yeast transformation were performed as described (Gietz and Schiestl, 1995). Yeast strain Y190 (MATα gal4 gal80 his3 trp-901 ade-101 ura-52 leu2-3, -112 + URA3::GAL → lacZ, LYS2::GAL → HIS3 cyh2) carrying bait plasmid pAS1-CYH2::UBC-1 was transformed with a random primed C. elegans cDNA library fused to GAL4 activation domain sequences (amino acids 768–881) in plasmid pACT (Elledge et al., 1991). A total of 2.1 × 10⁶ colonies were screened on 150-mm Trp-Leu-His SC + 35-mM 3-aminotriazole plates. The transformants were tested for β-galactosidase activity (Ausubel et al., 1998). False positive tests were performed in yeast strain Y187 as described (Durée et al., 1993). The pACT clones were rescued from yeast (Hoffman and Winston, 1987), electroporated into HB101 E. coli, purified, and sequenced with SEQEC 1 (ttc gat gat gaa acc cc) and SEQEC 2 (agg caa aac gat gta taa) primers. DNA sequences were searched for homologies to genes from C. elegans and other organisms using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul et al., 1997). The pACT clones were subjected to PCR with SEQEC 1 and an internal primer to R05D3.4 (gtg age ceg atc gtc gc) to identify clones encoding R05D3.4.

Yeast interaction trap studies

PCR was performed on pR1.7a with Vent DNA polymerase (New England Biolabs) and oligonucleotide primers EC 1 and EC 20 (tgg gat ccT TAG AAG GAC CAG GAC TGC TC). The ubc-1A-152 PCR product was cloned into the Ndel (5′) and BamHI (3′) sites of pAS1-CYH2 and sequenced.

Four GAL4 activation domain::RFP-1 truncation constructs in pACT2 were created by PCR with Pfu DNA polymerase (Stratagene) on rfp-1 in pACT. RFP-1 (1–183) was generated with oligos EC 11 (ccc ttc cat ggt gAT GAT GAA AAG TAA TGA AGG) and EC 23 (gcg aat tcC TAA CCA TTT GGA TTG AAG GTA GG) RFP-1 (184–313) was created with oligos EC 25 (get gtc cat ggG TGG TCG CAA TAA GGA GCT TAC AG) and EC 24 (ggc gat ccT TAT TCA AGA CGC AGA TTT TCG), RFP-1 (1–313) was generated with oligos EC 11 and EC 24, RFP-1 (408–839) was created with oligos EC 13 (get gcc cat ggc tAT GTC TGA AGA ACA TCA) and EC 14 (gcg aat cCT AGA TGA AAA TAC GAT GG). The four constructs were sequenced with SEQEC 1 and SEQEC 2 primers.

Y190 was singly transformed with the empty pACT2 vector, full-length RFP-1 in pACT, and RFP-1 (1–183), RFP-1 (184–313), RFP-1 (1–313), and RFP-1 (408–839) in pACT2. Each reaction was plated on a separate-Leu SC plate and incubated at 30°C for 3 days. Transformants were then transformed with pAS1-CYH2, pAS1-CYH2::UBC-1,
or pAS1-CYH2::UBC-1Δ152, plated on -Leu-Trp-His + 35-mM 3-aminotriazole SC plates, and tested for lacZ and His3 expression.

**Preparation of C. elegans protein extracts and Western blot analysis**

*C. elegans* Bristol strain (N2) was maintained at 15 or 20 °C as described (Brenner, 1974). Synchronous *C. elegans* populations were produced as described (Emmons et al., 1979; Sulston and Brenner, 1974). Protein extracts of five developmental stages (embryos, L1 stage, L2 stage, L3/L4 stage, and young adults) were prepared as described (Ding and Candido, 2000). Total protein (30 μg) for each developmental stage was loaded onto two 12.5% SDS polyacrylamide gels, separated by SDS-PAGE, and Coomassie blue stained. Western blot analyses with polyclonal RFP-1 (1:2500) or monoclonal actin (1:10,000) antibodies were performed as described (Ausubel et al., 1998).

**RFP-1 protein expression and purification**

Expression plasmid aa 1-329 RFP-1::pET28a or expression plasmid aa 408–775 RFP-1::pET28a was transformed into BL21 (DE3) bacteria, and protein expression was induced with 1 mM IPTG as described (Novagen pET Vector Manual). The insoluble RFP-1 truncation proteins were separated by SDS-PAGE and Coomassie blue stained. Soluble RFP-1 (1–329) protein was expressed by growing the bacteria at 30 °C and inducing protein expression overnight at 15 °C with 0.1 mM IPTG.

Inclusion bodies containing insoluble recombinant RFP-1 (aa 1–329 or 408–775) with a C-terminal 6 × His Tag were resuspended in 15 ml of denaturing buffer (50 mM sodium phosphate, pH 8.6, 300 mM NaCl, 6 M guanidine HCl) and purified on 2 ml of pre-equilibrated Talon Resin (Clontech) according to the manufacturer’s instructions.

**RFP-1 antibody production**

Polyclonal antibodies against RFP-1 were prepared as described (Harlow and Lane, 1988), in rabbits immunized with either amino acids 408–775 RFP-1 or amino acids 1–329 RFP-1. After purification on Talon Resin, the eluted antigen was dialyzed extensively at 4 °C against PBS (136 mM NaCl, 2.6 mM KCl, 10 mM Na2HPO4, 1.7 mM KH2PO4, pH 7.4) and fractionated by SDS-PAGE. The antigen bands were excised from the gels and used for immunizations.

**RFP-1 and UBC-1 co-immunoprecipitation studies**

Protein extracts of adult *C. elegans* were prepared by Dounce homogenizing animals in co-immunoprecipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, 2.5 mM PMSF). Each experiment contained 4 μg of recombinant UBC-1 (Leggett and Candido, 1997) and 50 μg of soluble RFP-1 (amino acids 1–329 with a C-terminal 6 × His tag) in a final reaction volume of 300 μl of co-immunoprecipitation buffer. Worm protein extract (50 μg), polyclonal anti-RFP-1 antibody (2.5 μg), and polyclonal anti-Hsp43 antibody (2.5 μg) were added as indicated and the mixtures were incubated (4 °C, 30 min). Next, 50 μl of 10% formalin-fixed *S. aureus* (Zymed) was added to the tubes and rocked (4 °C, 1 h). The *S. aureus* was collected by centrifugation (10,000 × g, 10 s) and washed twice with co-immunoprecipitation buffer. Protein-antibody complexes on the *S. aureus* pellet were eluted with 50 μl of Laemmli sample buffer, followed by incubation (37 °C, 10 min), and centrifugation (10,000 × g, 1 min). The supernatant was separated by SDS-PAGE, transferred to a PVDF membrane, and probed with a monoclonal UBC-1 antibody (1:4000).

**RFP-1 indirect immunohistochemical staining**

Indirect immunohistochemical staining of a mixed population of N2 animals was performed as described (Loer and Kenyon, 1993). *C. elegans* embryos were collected and subjected to indirect immunohistochemical staining as described (Goh and Bogaert, 1991).

**RNA interference against ubc-1 or rfp-1 by injection**

RNA interference (RNAi) against *ubc-1* or *rfp-1* by injection was performed as described (Fire et al., 1998). Plasmids pR1.7 and pBSIIKS::RFP-1 were used as templates to generate double-stranded *ubc-1* RNA (1.7 kb) and double-stranded *rfp-1* (2.6 kb including some 3’ UTR) RNA, respectively. Plasmid pBSIIKS::RFP-1 (nt 3–982) was used as a template to generate double-stranded RNA against the N-terminus of RFP-1.

**RNA interference against ubc-1 or rfp-1 by the feeding method**

RNA interference against *ubc-1* or *rfp-1* was performed as described (Kamath et al., 2000; Timmons et al., 2001). To produce a *ubc-1* template for bidirectional transcription, the *ubc-1* coding sequence was cloned into the XbaI and *Hind*III sites of pPD129.36 (Timmons et al., 2001), and named pPD129.36::UBC-1. A *Xhol* fragment of the *rfp-1* cDNA (corresponding to nt 3–982) was cloned into pPD129.36 and named pPD129.36::RFP-1 (nt 3–982). As a negative control, 5–10 L4 hermaphrodite animals were raised on HT115 (DE3) bacteria transformed with empty pPD129.36 vector (no exposure to double-stranded RNA). As a positive control, five N2 L4 hermaphrodites were raised on HT115 (DE3) bacteria transformed with...
pPD129.36::UBC-2, and the F1 progeny were examined for embryonic lethality.

RNA extraction and RT-PCR of C. elegans exposed to double-stranded ubc-1 RNA

N2 animals were raised at 20°C on seven Petri plates spread with HT115 (DE3) bacteria transformed with either pPD129.36::UBC-1 or empty pPD129.36 vector. Total RNA was extracted with TRIZOL (Invitrogen Life Technologies) as described (Burdine and Stern, 1996) with the following modifications: 50 µl of packed animals were resuspended in 800 µl of TRIZOL, and following extraction with chloroform, the RNA in the aqueous layer was precipitated with 160 µl of isopropanol.

RT-PCR with Ready-to-Go-RT-PCR beads (Amersham Pharmacia) was performed on 500 ng of total RNA with oligonucleotide primers EC 100 (ctt ttc cca tca tcc ccg t) and EC 101 (agg ccg aaa atc ega aaa) to amplify a 394 bp fragment corresponding to the 3' untranslated region of ubc-1. RT-PCR was performed on 500 ng of total RNA with oligonucleotide primers R09-5 (cgg gat ccA TGT TCA ATC TTC AAA AAC GAA TCA ACG GCA ACA AC) and R09-3 (ccc aag cTT AGC AAT ATC TAG AAA TGT ATT CC) corresponding to the 543 bp coding region.

Fig. 1. Conservation of C. elegans RFP-1 in eukaryotes. Clustal W alignment of RFP-1 sequence homologues in R. norvegicus (Staring), H. sapiens (KIAA0661), and S. cerevisiae (Bre-1). Black shading, identical residues. Gray shading, conserved residues. Asterisks indicate the positions of the conserved C-terminal C3HC4 RING finger’s cysteiny1 and histidyl residues that likely coordinate two zinc atoms.
ubc-12. The RT-PCR products were separated by agarose gel electrophoresis.

**Results**

**Identification of proteins that interact with C. elegans UBC-1**

A randomly primed *C. elegans* cDNA library was screened using UBC-1 as a bait in a yeast two-hybrid screen (Chien et al., 1991; Fields and Song, 1989). Of the 62 positive cDNA clones obtained in this screen, 77% encoded R05D3.4, a novel RING finger protein located on chromosome III. R05D3.4 was renamed RFP-1 for RING finger protein 1. Plasmid pACT 38 contained an almost full-length cDNA consisting of bp 3–2520 of the predicted *rfp-1* open reading frame as well as 103 bp of 3′ untranslated region. The predicted *rfp-1* coding region consists of 2520 bp and upon expression produces an 839 amino acid protein with a predicted molecular weight of 97.6 kDa and a pKᵢ of 6.87. The DNA sequence obtained from directly sequencing the *rfp-1* cDNA isolated from the yeast two-hybrid screen revealed a six bp insertion (two extra amino acids) and a nine bp insertion (three extra amino acids). This suggests that the GenBank coding sequence as described contains incorrectly assigned exons, specifically exons six and seven. The SMART program (Letunic et al., 2002; Schultz et al., 1998) predicted six α-helical regions, a “hidden” (poor match to the consensus sequence) basic region leucine zipper, and a C3HC4 RING finger domain.

WormBase (http://www.wormbase.org, release WS99, April 26, 2003; Harris et al., 2003; Stein et al., 2001) listed 162 predicted RING finger proteins encoded by the *C. elegans* genome. BLAST analysis (Altschul et al., 1997) yielded no clear RFP-1 relatives in *C. elegans*, that is, RFP-1 did not appear to belong to a protein family. BLAST analysis of the RFP-1 sequence showed that it was 48% similar to a 1002 residue *R. norvegicus* RING finger protein called Staring or Rnf40. RFP-1 shared 48% similarity and 23% identity to the human ortholog of Staring, a 1001 residue *H. sapiens* protein K1AA0661. *S. cerevisiae* Bre1p was 40% similar and 21% identical to RFP-1. All of these proteins contain RING finger domains important for protein–protein interactions (Fig. 1).

Fourteen percent of the cDNAs recovered from the yeast two-hybrid screen encoded C32E8.11, a homologue of *S. cerevisiae UBR1* and *M. musculus* E3α (Kwon et al., 1998). Ubr1p is a RING finger ubiquitin-protein ligase (E3) that interacts with Rad6p, the yeast homologue of UBC-1 (Dohmen et al., 1991). Ubr1p multiubiquitylates and targets for degradation the transcriptional repressor Cup9p (Turner...
et al., 2000) and the cohesin Scc1p via the N-end rule pathway (Bachmair and Varshavsky, 1989; Bachmair et al., 1986; Rao et al., 2001).

Mapping the UBC-1 and RFP-1 interaction: yeast interaction trap studies

In order to map the region of interaction between UBC-1 and RFP-1, studies were performed with either full-length UBC-1 or a mutated UBC-1 lacking its acidic C-terminal tail, UBC-1Δ152. The two UBC-1 baits were assayed individually for their ability to interact with the full-length GAL4 activation domain::RFP-1 fusion protein or with each of four GAL4 activation domain::truncated RFP-1 fusion proteins. The RFP-1 truncations were chosen based on helical domains identified by the SMART program (Letunic et al., 2002; Schultz et al., 1998). These five constructs consisted of full-length RFP-1, Helix 1 alone (RFP-1 1–183), Helix 2 alone (RFP-1 184–313), Helices 1 and 2 together (RFP-1 1–313), and Helices 5 and 6 with the C3HC4 RING finger domain (RFP-1 408–839). Expression of the UBC-1Δ152 bait and the five RFP-1 prey constructs in yeast were confirmed by Western blotting (data not shown).

Fig. 2 summarizes the results of the yeast interaction trap experiments. None of the GAL4 activation domain::RFP-1 fusion proteins independently activated His3 or lacZ transcription. None of the GAL4 activation domain::RFP-1 fusion proteins interacted with empty pAS1-CYH2, indicating that the RFP-1 interaction was specific to UBC-1. Similarly, neither the UBC-1 nor the UBC-1Δ152 baits could interact with empty pACT2 vector, indicating that the interaction was RFP-1-dependent. Full-length RFP-1 interacted with both UBC-1 and UBC-1Δ152. Thus, the acidic C-terminal tail of UBC-1 was dispensable for the interaction with RFP-1. Neither the first 183 amino acids (Helix 1 alone), nor amino acids 184–313 of RFP-1 (Helix 2 alone) were sufficient for interaction with UBC-1. The N-terminus of RFP-1 (1–313) was, however, sufficient for interaction with UBC-1, while the C-terminal domain (408–839) did not appear to interact directly with UBC-1. The N-terminus of RFP-1 (1–313) was also capable of interactions with UBC-1Δ152, thereby demonstrating that the acidic tail of UBC-1 is dispensable for this interaction.

Recombinant UBC-1 and RFP-1 co-immunoprecipitate

Several attempts were made to express soluble, full-length RFP-1 (amino acids 1–839) in E. coli, insect cell lines Kcl, Sf9, and High Five, and also in rabbit reticulocyte lysates. Full-length RFP-1 was not expressed in any of the insect cell lines studied, nor was it expressed in rabbit

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<table>
<thead>
<tr>
<th>GAL4 Activation Domain Construct</th>
<th>GAL4 DNA Binding Domain Construct</th>
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<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>β-gal*</td>
</tr>
<tr>
<td>Empty pACT2 Vector</td>
<td>-</td>
</tr>
<tr>
<td>RFP-1</td>
<td>-</td>
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<tr>
<td>aa 1-313</td>
<td>-</td>
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<tr>
<td>aa 1-183</td>
<td>-</td>
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<tr>
<td>aa 184-313</td>
<td>-</td>
</tr>
<tr>
<td>aa 408-839</td>
<td>-</td>
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</table>

* β-galactosidase activity  
** Growth on histidine dropout medium containing 35 mM 3-amin0-1, 2, 4 triazole
reticulocyte lysates at significant levels. While *E. coli* expressed small quantities of full-length RFP-1, the protein was entirely insoluble (data not shown). Our inability to express soluble RFP-1 limited functional studies. However, a 38-kDa soluble RFP-1 truncation protein, RFP-1 (1–329 with a C-terminal 6 × His tag), was expressed in *E. coli*. This recombinant protein was used in co-immunoprecipitation studies as an independent approach to study the interaction between UBC-1 and RFP-1.

Each experiment contained recombinant UBC-1 and RFP-1 (amino acids 1–329 with a C-terminal 6 × His Tag). *C. elegans* protein extract and either polyclonal anti-RFP-1 antibody or a control polyclonal anti-Hsp43 antibody were added as indicated in Fig. 3. RFP-1 antibodies co-immunoprecipitate UBC-1 and RFP-1 in buffer (lane 2) as well as in *C. elegans* extract (lane 3). In the negative control, Hsp43 antibodies did not co-immunoprecipitate UBC-1 and RFP-1 (lanes 4 and 5). The observation that RFP-1 antibodies could co-immunoprecipitate UBC-1 with the N-terminus of RFP-1 (1–329) confirmed the yeast interaction trap studies (Fig. 2), in which RFP-1 (1–313) was sufficient to interact with UBC-1.

**RFP-1 is a nuclear protein in *C. elegans* embryos, larvae, and adults**

Protein extracts from five *C. elegans* developmental stages were analyzed by Western blotting for the presence of the 97.6-kDa RFP-1 using anti-RFP-1 antibodies. Fig. 4A shows that RFP-1 is expressed in all life stages of *C. elegans*. RFP-1 expression is high in embryos, L1, and L2 animals (lanes 1–3) and appears to decrease slightly in the L4 (lane 4) and young adult (lane 5) stages. Extracts of the L2 larval stage appear to have enhanced proteolytic activity; the cause of which is unknown. As a control for protein loading, the extracts from the five developmental stages were probed with a monoclonal antibody against actin (48 kDa) (B).

Fig. 4. RFP-1 is expressed in all life stages of *C. elegans*. Protein extracts of *C. elegans* embryos (E), larval stages 1–4 (L1–L4), and young adults (YA) were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with polyclonal antibodies generated against the RFP-1 C-terminal region (A). As a control for protein loading, the protein extracts from the five developmental stages were probed with a monoclonal antibody against actin (48 kDa) (B).
staining was also observed in pharyngeal and intestinal cell nuclei in the head region of the adult animal (data not shown).

**RNA interference against ubc-1 or rfp-1 in C. elegans**

RNA interference (RNAi) is a reverse-genetics tool used to study gene function in *C. elegans* (Fire et al., 1998; Montgomery et al., 1998). RNA interference experiments were performed by either injecting double-stranded *ubc-1* RNA into hermaphrodites or by feeding hermaphrodites bacteria expressing double-stranded *ubc-1* RNA. RNAi against *ubc-1* in either N2 animals or in an RNA interference-sensitive strain called *rrf-3* (Simmer et al., 2002) had no deleterious effects on embryonic viability, that is, embryos hatched as L1 animals with normal developmental timing. Moreover, these F1 animals progressed through the four larval stages to become egg-laying adults, with no obvious abnormalities (Table 1 and data not shown).

To eliminate the possibility that the RNAi feeding method was not eliminating endogenous *ubc-1* mRNA, RT-PCR was performed on total RNA extracted from animals raised on bacterial lawns expressing double-stranded *ubc-1* RNA. These animals had undetectable levels of endogenous *ubc-1* RNA (Fig. 6, lane 2). In the control experiments, animals that were not exposed to *ubc-1* RNA...
interference contained endogenous *ubc-1* RNA (lane 1). Also, RT-PCR on total RNA extracted from either control animals or animals exposed to double-stranded *ubc-1* RNA had readily detectable levels of endogenous *ubc-12* RNA (lanes 3 and 4). Heat inactivation of reverse transcriptase, followed by PCR for *ubc-1*, produced a weak signal, indicating that there was virtually no contaminating DNA (lane 5). The absence of a *ubc-1* RNAi-induced phenotype was also observed in a genome-wide RNAi screen by Kamath et al. (2003).

When *rrf-3* or *N2* hermaphrodites were injected with full-length *rfp-1* double-stranded RNA, 71% of *rrf-3* progeny and 74% of *N2* progeny arrested at the L1 stage as sluggish, abnormal L1 larvae that eventually died (Table 1). These larvae often had abnormal tail morphology (Fig. 7B). To eliminate the possibility that the L1 arrest was due to interference with other RNAs encoding essential RING finger domains, *N2* animals were injected with double-stranded RNA corresponding to the N-terminus of *rfp-1* (nt 3–982). This *rfp-1* RNA does not encode the RING finger domain of RFP-1. Again, 65% of these *N2* progeny arrested as abnormal L1 animals (Table 1). The animals that escaped the L1 arrest were developmentally delayed, and this slow growth phenotype was confirmed by Kamath et al. (2003). Interestingly, these animals developed everted vulvas and egg-laying defects, as described below.

We also used the feeding method to expose animals to *rfp-1* double-stranded RNA. As a positive control for the feeding method, *N2* hermaphrodites were raised on bacteria expressing double-stranded *ubc-2* RNA, and the F1 generation was scored for embryonic lethality (Jones et al., 2001). One hundred percent of the embryos laid by *N2* hermaphrodites exposed to *ubc-2* double-stranded RNA failed to hatch (data not shown).

In contrast to the injection experiments (Table 1), feeding *N2* hermaphrodites double-stranded *rfp-1* (nt 3–982) RNA did not cause a severe L1 arrest in the progeny. Only 2% of the progeny arrested at the L1 stage. While many of the larvae eventually became egg-laying adults, approximately 10% of the young adults had everted vulvas (*evl*) (Table 1). The *evl* animals often burst, extruding intestinal and gonadal tissues from the body cavity (Figs. 7C, D). Approximately 5% of the adults had egg-laying defects (*egl*) characterized by a bloated appearance (a bag of eggs). These animals laid few eggs (Table 1).

To determine if simultaneous loss of *rfp-1* and *ubc-1* function exacerbates the observed *rfp-1* phenotypes, *rrf-3* or *N2* animals were injected with both *ubc-1* and *rfp-1* double-stranded RNAs. RNAi against *ubc-1* plus *rfp-1* resulted in a slightly lower frequency of L1 arrest compared to the single *rfp-1* RNA injection (Table 1). This is most likely due to a dilution of the *rfp-1* RNA in these double injection experi-

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**Table 1**

Summary of the phenotypes of the progeny of *rrf-3* or *N2* hermaphrodites exposed to either *rfp-1* (3–982), full-length *rfp-1*, or *ubc-1* double-stranded RNAs.

<table>
<thead>
<tr>
<th>Strain</th>
<th>dsRNA</th>
<th>RNAi method</th>
<th>Embryos (n)</th>
<th>% L1 arrest</th>
<th>% evl</th>
<th>% egl</th>
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<tbody>
<tr>
<td><em>rrf-3</em></td>
<td>Nonea</td>
<td>Injection</td>
<td>107</td>
<td>0 (0)b</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>rrf-3</em></td>
<td><em>rfp-1</em></td>
<td>Injection</td>
<td>396</td>
<td>71 (281)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>rrf-3</em></td>
<td><em>ubc-1 + rfp-1</em></td>
<td>Injection</td>
<td>155</td>
<td>66 (102)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>N2</em></td>
<td>Nonea</td>
<td>Injection</td>
<td>127</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>N2</em></td>
<td><em>rfp-1</em></td>
<td>Injection</td>
<td>249</td>
<td>74 (185)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>N2</em></td>
<td><em>ubc-1 + rfp-1</em></td>
<td>Injection</td>
<td>179</td>
<td>76 (136)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>N2</em></td>
<td><em>rfp-1</em> (3–982)</td>
<td>Injection</td>
<td>320</td>
<td>65 (209)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>N2</em></td>
<td><em>ubc-1 + rfp-1</em> (3–982)</td>
<td>Injection</td>
<td>199</td>
<td>36 (72)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>N2</em></td>
<td>Vectorc</td>
<td>Feeding</td>
<td>957</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>N2</em></td>
<td><em>ubc-1</em></td>
<td>Feeding</td>
<td>700</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>N2</em></td>
<td><em>rfp-1</em> (3–982)</td>
<td>Feeding</td>
<td>1663</td>
<td>2 (32)</td>
<td>10 (168)</td>
<td>5 (79)</td>
</tr>
</tbody>
</table>

n (number); ND (not determined); *evl* (everted vulva); *egl* (egg-laying defective).

a Control experiments in which animals were not injected with double-stranded RNA.
b The actual number of animals with the indicated phenotype is shown in parentheses.
c Control experiments in which animals were raised on HT115 (DE3) bacteria transformed with the empty pPD129.36 vector.
ments. Clearly, the *rfp-1* RNAi phenotype was not enhanced by simultaneous targeting of *ubc-1*.

**Discussion**

In this yeast two-hybrid screen, RFP-1 (R05D3.4) and UBR1 (C32E8.11) were isolated as potential binding partners of UBC-1. The interaction between UBC-1 and RFP-1, a 97.6-kDa C3HC4 RING finger protein, was recently detected in proteome-wide yeast two-hybrid screens for proteins involved in the DNA damage response in *C. elegans* (Boulton et al., 2002).

Proteins containing RING finger domains have been shown to possess ubiquitin-protein ligase activity (Lorick et al., 1999), with the RING finger domain usually recruiting and activating the ubiquitin-conjugating enzyme (Joazeiro et al., 1999). The RING finger family of E3s is diverse and includes single subunit E3s that recognize and bind to sequence motifs in their target proteins, and multi-subunit E3s such as the SCF or APC that have a RING finger protein as an important component (Jackson et al., 2000).

The function of RFP-1 in *C. elegans* is unknown. RFP-1 could be a ubiquitylation substrate of UBC-1, a positive or negative regulator of UBC-1, or a RING finger ubiquitin-protein ligase that works in concert with UBC-1 to target specific proteins for ubiquitylation. In addition to the cysteinyl residues in the RING finger domain, RFP-1 contains 10 cysteinyl residues that could potentially form thiolesters with ubiquitin, similar to the hect E3s. In the absence of substrates, ubiquitylations on E3s have been observed (Joazeiro et al., 1999; Lorick et al., 1999; Pickart, 2001). Unfortunately, E3 assays with RFP-1 were impeded by the inability to express soluble full-length RFP-1. In assays with recombinant UBC-1 and the RFP-1 (1–329) truncation protein, no ubiquitylation of RFP-1 was observed (data not shown). However, since the recombinant RFP-1 (1–329) was truncated, these experiments do not exclude the possibility that intact RFP-1 is a functional E3 in vivo.

The N-terminus of RFP-1 (1–313), containing the first two helical regions, was required for the interaction with UBC-1 (Fig. 2). These regions may be required for stability and folding of RFP-1, such that the interaction with UBC-1 is obliterated if either helix is missing. While RING finger domains have been reported to recruit ubiquitin-conjugating enzymes (Lorick et al., 1999), the RING finger of RFP-1 did not function in this capacity. In *S. cerevisiae*, Rad6p binds to the basic rich region (BRR) of its cognate E3 Ubr1p, and the

![Fig. 7. RNA interference against *rfp-1* produces an L1 arrest and an abnormally everted vulva phenotype.](image)
RING finger domain of Ubr1p was dispensable for this interaction (Xie and Varshavsky, 1999). Although RFP-1 and Ubr1p share no sequence similarity outside of the RING finger domain, it is interesting that both of these proteins contain basic residue rich regions that recruit the same UBC homologue (UBC-1 or Rad6p, respectively). Perhaps the C-terminal RING finger domain of RFP-1 interacts with other unidentified protein targets.

The N- or C-terminal extensions of UBcs can often confer substrate specificity, modulate ubiquitylation activity, promote self-association, or direct subcellular localization of a given UBC (Block et al., 2001; Haldeman et al., 1997; Leggett and Candido, 1997). RFP-1 interacted with UBC-1Δ152, indicating that the acidic C-terminal tail of UBC-1 was dispensable for the interaction. This argues against a simple electrostatic attraction as the basis of the interaction and implies that residues within the core domain of UBC-1 are required.

RFP-1 antibodies co-immunoprecipitated recombinant UBC-1 and RFP-1 (1–329) in either buffer or C. elegans extract (Fig. 3). These results show that ATP and ubiquitin are not required for the interaction between UBC-1 and RFP-1. However, ATP and/or ubiquitin may increase the interaction in C. elegans extract by posttranslationally modifying either protein. UBC-1 itself is stably monoubiquitylated on Lys 162 (Leggett and Candido, 1997), and it is possible that this modification is important for interactions with other proteins such as RFP-1. However, in this experiment (Fig. 3, lane 2 vs. 3), there is no difference in the mobilities of UBC-1 and thus, no evidence of monoubiquitylation of UBC-1 being correlated with RFP-1 binding.

The interaction between UBC-1 and RFP-1 appeared to be stronger in C. elegans extract than in buffer perhaps due to helpful chaperone activity in the extract that promoted proper folding of the added recombinant UBC-1 and RFP-1 proteins. Native UBC-1 in the extract may have been co-immunoprecipitated with RFP-1. Another interpretation is that the protein concentration of the C. elegans extract (0.2 mg/ml) was high enough to contribute to “molecular crowding” that increased the association between UBC-1 and RFP-1 (Ellis, 2001). Attempts to co-immunoprecipitate endogenous RFP-1 and UBC-1 from C. elegans extract were unsuccessful, presumably due to low-protein concentrations which would make detection difficult.

The expression profile of RFP-1 (Fig. 4) was similar to that of ubc-1 mRNA (Leggett, 1996). UBC-1 and RFP-1 could therefore function together throughout development. Indirect immunohistochemical staining shows that RFP-1 is a nuclear protein in embryos, larvae, and adults (Fig. 5). RFP-1 contains several putative nuclear localization sequences in its N-terminal region including QKRRKIQ (residues 24–30), RSKRRQ (residues 77–82), KRRK (residues 25–28), RKKR (residues 218–221), and KRIAELKERNASKRQ (residues 66–82) (Kieren et al., 1990; Nakai and Kanehisa, 1992; Stephen et al., 1997). RFP-1 is presumably required in the nucleus, where it may act as a RING finger ubiquitin-protein ligase that works in concert with UBC-1 to target specific nuclear proteins for ubiquitylation.

As yet, there exist no mutants of ubc-1 in C. elegans. No obvious phenotype was induced by RNA interference against ubc-1 (Jones et al., 2001; Table 2). RT-PCR on animals exposed to ubc-1 RNA interference showed that endogenous ubc-1 RNA was eliminated and confirms that the RNAi method employed was efficient (Fig. 6). Animals exposed to ubc-1 RNA produced fertilized embryos that subsequently developed into healthy larvae, suggesting that ubc-1 is not required for spermatogenesis in C. elegans. This differs from the role of RAD6 homologues in spermatogenesis in other multicellular organisms (Roest et al., 1996).

The absence of a ubc-1 RNA interference-induced phenotype may be due to compensation by other ubiquitin-conjugating enzymes. There are 20 ubiquitin-conjugating enzymes in C. elegans, and it is possible that one or more of them is capable of substituting for UBC-1. Perhaps UBC-3 and UBC-8, which have acidic carboxy-terminal extensions, could functionally substitute for UBC-1 (Jones et al., 2001).

RNAi against rfp-1 had no visible effect on embryonic viability, suggesting that rfp-1 is not required for embryogenesis (data not shown). The lack of an embryonic phenotype was confirmed by Gonczy et al. (2000) in their RNA interference experiments on approximately 96% of the 2315 predicted open reading frames on chromosome III. Interestingly, injection of double-stranded rfp-1 RNA produced a severe L1 arrest in the F1 progeny. Animals that managed to escape this L1 arrest had a slow growth phenotype, but eventually developed into adults that had everted vulvas and egg-laying defects (Table 1 and Fig. 7). By contrast, feeding animals double-stranded rfp-1 RNA did not produce a severe L1 arrest, although animals were egl and evl (Table 1). RNA interference by injection may have delivered a more concentrated sample of double-stranded rfp-1 RNA to target rfp-1 at the L1 stage. Kamath et al. (2000) observed that the feeding method of RNA interference was particularly useful for detecting secondary phenotypes of essential genes.

Our results contradict the findings of Gonczy et al. (2000), who failed to detect an rfp-1 RNAi-inducible phenotype in L1 larvae or adults. Kamath et al. (2003) also failed to detect the L1 arrest and evl and egl rfp-1 RNAi-induced phenotypes, although they confirmed our observation that rfp-1 RNAi is associated with slow growth. These evl and egl phenotypes may have been overlooked due to the high-throughput approach of their experiments.

The evl phenotype suggests that rfp-1 has a role in maintaining proper vulval structure or development. The specification and proliferation of vulval cells and vulval morphogenesis were not examined in detail in animals exposed to rfp-1 RNAi. The vulva appeared to develop normally in animals exposed to rfp-1 RNA interference, as several animals had the “Christmas tree” structure of fused vulval cells (data not shown). Although the function of rfp-1 in vulval development is unknown, one possibility is that...
RFP-1 could be an E3 that targets specific regulators of vulval development for ubiquitylation.

Interestingly, the rfp-1 RNAi-inducible phenotype is similar to some of the secondary phenotypes produced by RNAi against the ubiquitin-conjugating enzymes ube-2, ube-9, or ube-12 (Jones et al., 2001). Specifically, RNAi against ube-9, ube-12, or rfp-1 induces vulval eversion and subsequent rupture of the animals. Egg-laying defects arose in F1 animals exposed to rfp-1 or ube-9 RNA. These similar phenotypes may indicate that rfp-1 could be a ubiquitin-protein ligase that has similar targets as those of ube-2, ube-9, and ube-12. In this view, lack of RFP-1 would remove the specificity factor that controls ubiquitylation of substrates and would produce phenotypes such as L1 arrest or vulval eversion. By contrast, lack of UBC-1 could be rescued by other UBCs (perhaps UBC-3 or UBC-8) and no obvious null phenotype would be discernible.

In *S. cerevisiae*, Rad6p-dependent ubiquitylation of histone H2B (Robzyk et al., 2000) is required for methylation of histone H3 and subsequent gene silencing (Dover et al., 2002; Sun and Allis, 2002). Significantly, Bre1p, the *S. cerevisiae* homolog of RFP-1 (Fig. 1), was recently shown to be a RING finger E3 required for the Rad6p-dependent monoubiquitylation of histone H2B and subsequent methylation of histone H3 (Hwang et al., 2003). Given their similarity to RAD6 and BRE1, respectively, it would be interesting to ascertain if ubc-1 and rfp-1 are required for histone ubiquitylation or germine-specific gene silencing in *C. elegans*.

Although the function of RFP-1 in *C. elegans* remains elusive, a recent study demonstrated that Staring, the rat homologue of RFP-1 (Fig. 1), is a ubiquitin-protein ligase for the neuronal membrane protein Syntaxin 1. The 1002 residue Staring protein (114 kDa) was 26% identical and 38% similar to *C. elegans* RFP-1, and both proteins share a similar domain structure composed of six helical regions and a C-terminal C3H4 RING finger (Chin et al., 2002). *C. elegans* unc-64 encodes syntaxin that is required for synaptic transmission, as unc-64 animals have uncoordinated movement, are developmentally delayed and have increased acetylcholine levels. UNC-64 is present in the nerve ring, ventral and dorsal nerve cords, and in certain secretory cells such as intestinal, spermathecal, and vulval (uv1 cell) cells (Ogawa et al., 1998; Saifee et al., 1998). Our studies show that RFP-1 is localized to the nerve ring and to intestinal cells, where RFP-1 may target UNC-64.

We propose a model in which RFP-1 is a single subunit RING finger ubiquitin-protein ligase that interacts with UBC-1 to perhaps target Syntaxin 1 as well as other unidentified substrates for ubiquitylation in *C. elegans*.

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