The NED-8 Conjugating System in Caenorhabditis elegans Is Required for Embryogenesis and Terminal Differentiation of the Hypodermis

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This work has identified the enzymes involved in the activation and conjugation of the ubiquitin-like protein NED-8 in Caenorhabditis elegans. A C. elegans conjugating enzyme, UBC-12, is highly specific in its ability to utilize NED-8 as a substrate. Immunostaining shows that NED-8 is conjugated in vivo to a major target protein with a conjugate size of 90 kDa. While the amount of this conjugate is developmentally regulated with reduced levels in the larval stages, the mRNA encoding C. elegans UBC-12 is constitutively produced throughout development, as is NED-8 itself. The importance of the NED-8 conjugating system in C. elegans was determined by RNA interference (RNAi) assays using double-stranded RNA encoding NED-8, UBC-12, or the NED-8 activating enzyme component ULA-1. The progeny of both ned-8 and ubc-12 RNAi-treated hermaphrodites either arrested during embryonic development or underwent abnormal postembryonic development. The effect on postembryonic development was pleiotropic, the most frequent gross abnormality being vulval eversion during the L4 stage. Individuals with an everted vulva either burst at the L4 to adult molt or gave rise to adults incapable of egg laying. Additionally, both ned-8 and ubc-12 RNAi induced a striking abnormality in the alae, structures produced by the lateral hypodermal seam cells in the adult nematode. Affected alae were patchy and frequently diverged around a central space. Vulval defects were also produced by RNAi directed at C. elegans ula-1. This is the first demonstration of a requirement for NED-8 conjugation in metazoan development. © 2000 Academic Press

Key Words: C. elegans; NED-8; NEDD8; UBC-12; hypodermis; cullin; alae; vulva; RNAi; ULA-1.

INTRODUCTION

The conjugation of ubiquitin to other polypeptides has emerged as a key step in targeting a wide variety of proteins for degradation (Ciechanover, 1994; Haas and Siepmann, 1997; Hershko and Ciechanover, 1992; Hochstrasser, 1996). Ubiquitin, a highly conserved 76-residue polypeptide, is usually conjugated to targets through an isopeptide bond between its C-terminal glycyl residue and an ε-amino group on the target protein. The ubiquitin moiety itself contains lysyl residues which can serve as points of attachment for ubiquitin, thus leading to the formation of multiubiquitin chains of varying lengths. Multibu ubiquitinates proteins are recognized by the 26S proteasome, and this generally re-...
Adding to the complexity of the ubiquitin system is the existence of several ubiquitin-like proteins. Some of these are covalently linked to target proteins but do not act as a signal for degradation. In mammals, UCRP (ubiquitin cross-reactive protein) is an interferon-inducible protein consisting of two tandem ubiquitin domains (Haas et al., 1987). UCRP is conjugated to a number of unidentified intracellular proteins, through an apparently distinct enzymatic pathway (Narasimhan et al., 1996), and the modified proteins associate with the cytoskeleton (Loeb and Haas, 1994). A different ubiquitin-like protein, variously known as sentrin, SMT3, SUMO-1, PIC1, UBL1, and GMPI (Johnson and Hochstrasser, 1997), is conjugated to the nuclear Ras-like GTPase, ranGAP1 (Mahajan et al., 1997), is conjugated to the nuclear pore complex. Genetic and biochemical studies in Saccharomyces cerevisiae have elucidated the role of a third such protein, RUB1 (Lammer et al., 1996) and serves as a signal for the association of the VCB ring-finger protein RBX1 (Feldman et al., 1997; Kamura et al., 1999). CDC53 is a member of a gene family known as cullins, with homologs in other eukaryotes. Caenorhabditis elegans has at least five cullin-encoding genes (Kipreos et al., 1996). Mammalian CUL1, the ortholog of yeast CDC53, is also known to assemble into an SCF complex (Lisztwan et al., 1998), while mammalian CUL2 was recently shown to be part of another ubiquitin ligase complex called the VCB (Lisztwan et al., 1999). The VCB complex consists of the proteins SKP1 and CDC53, the conjugating enzyme CDC34, an F-box protein, and the ring-finger protein RBX1 (Feldman et al., 1997; Kamura et al., 1999). CDC53 is a member of a gene family known as cullins, with homologs in other eukaryotes. Caenorhabditis elegans has at least five cullin-encoding genes (Kipreos et al., 1996). Mammalian CUL1, the ortholog of yeast CDC53, is also known to assemble into an SCF complex (Lisztwan et al., 1998), while mammalian CUL2 was recently shown to be part of another ubiquitin ligase complex called the VCB (Lisztwan et al., 1999). The VCB complex in mammals contains elongin B; elongin C; CUL2; one of the conjugating enzymes UBCH5a, b, or c; and RBX1 (Iwai et al., 1999; Kamura et al., 1999).

Importantly, RUB1-deficient yeast are normal, although a synthetic lethality occurs in RUB1-deficient strains carrying temperature-sensitive alleles of cdc34, the E2 component of the SCF (Lammer et al., 1998). This suggests that the RUB1 modification of CDC53 somehow stabilizes or assists in the formation of the complex, perhaps by affecting the docking of CDC53 and CDC34. The RUB1 homolog in mammals, NEDD8 (neural precursor cell-expressed, developmentally down-regulated; Kumar et al., 1993), is conjugated to all six of the mammalian cullins (Hori et al., 1999; Liakopoulos et al., 1999) although the phenotype of a NEDD8 deficiency in mammals is currently unknown.

The C. elegans gene encoding NED-8 was identified as part of the genome sequencing project (Waterston and Sulston, 1995). In an effort to elucidate the function of this widely distributed ubiquitin-like protein, we have used heterologous expression, immunological techniques, and double-stranded RNA-mediated gene interference (RNAi) (Fire et al., 1998) to study the biological role of NED-8 in C. elegans. RNAi is a powerful new approach to the study of gene function in C. elegans that was unveiled by the discovery that double-stranded RNA complementary to a gene sequence could produce a phenocopy of a true genetic deletion (Fire et al., 1998). In this technique, double-stranded RNA, prepared from the sequence of the mature gene transcript (either coding or noncoding regions but exclusive of intron sequences) is injected into hermaphrodites, and the progeny of the injected individual are examined for phenotypic abnormalities. The effect is both highly specific for the gene of interest and highly penetrant, but does not generally persist in the germ line. Many C. elegans genes have been analyzed using this technique including the syntaxins (Jantsch-Plunger and Glotzer, 1999), the chromosome segregation protein HCP-1 (Moore et al., 1999), and components of the dynactin complex (Skop and White, 1998). The RNAi technique has also been successfully applied in other organisms including Drosophila (Tuschl et al., 1999) and zebrafish (Li et al., 2000).

**MATERIALS AND METHODS**

**Cloning, Expression, and Purification of C. elegans NED-8**

A recombinant C. elegans NED-8 clone was produced by the polymerase chain reaction (PCR) using oligonucleotides 5'-agtcatagcgtcatcacaaggaac and 5'-gaagcttacctccgaagacaagac. The target DNA for the PCR was cDNA clone T01966 (McCombie et al., 1992). The reaction included 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl, pH 8.75, 2 mM MgSO₄, 0.1% Triton X-100, 100 μg/ml BSA, 50 pmol of each oligonucleotide, 10 ng of clone T01966, and 0.5 units of Pfu DNA polymerase (Stratagene). The resulting PCR product was cloned into the vector pRSET A (Invitrogen) between the NdeI and the HindII sites in the polylinker. This cloning strategy eliminated the polyhistidine domain from the vector. A similar strategy, using a 5'-oligonucleotide with a synthetic BamHI site, was used to produce an amino-terminal His₆-tagged version of NED-8.

The pRSET clone was transformed into Escherichia coli BL21(DE3) cells (Novagen). Scrapings of freshly transformed cells were inoculated into enriched medium (10 g Bacto-Tryptone, 10 g yeast extract, and 10 g NaCl per liter) containing 200 μg/ml ampicillin. Upon reaching an optical density of 0.6, the cells were induced with 1 mM IPTG and harvested by centrifugation 2 h later. The bacterial cell pellet from 500 ml of culture was resuspended in 30 ml of 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and three protease inhibitor tablets (Complete, Mini; Boehringer Mannheim) were added, yielding a final EDTA concentration of 1 mM.

2 To be consistent with C. elegans gene nomenclature, we have designated the C. elegans gene ned-8 and the corresponding protein NED-8. Similarly, UBC-12, ULA-1, and UBA-3 are hyphenated in C. elegans nomenclature.
The mixture was sonicated at 5°C using a Heat Systems Model XL sonifier with standard microtip at power setting 3, for a total of 15 min in 10-s bursts with 20-s cooling intervals. The solution was then centrifuged at 10,000g for 10 min at 5°C. The supernatant was precipitated with ammonium sulfate in stages (25, 40, 60, 75, and 100% saturation). At each stage, the solution was stirred on ice for 15 min, and the precipitate was removed by centrifugation at 10,000g for 10 min. The supernatant remaining after the 100% precipitation step was dialyzed for 16 h in two changes of 4 L of 50 mM sodium phosphate buffer, pH 6.5, with 1 mM DTT and 1 mM EDTA. The dialyzed sample was concentrated using an Amicon stirred cell with YM 3 Diaflo membranes (Amicon). The purity of the final product depended on the concentration of cells used in the sonication, and no more than 500 ml of culture could be processed in a 30-ml sonication volume.

The concentration of recombinant NED-8 was determined by BCA (bicinchoninic acid) assays (Pierce) as Coomassie based assays gave equivocal results. Similarly, NED-8 was stained on SDS-PAGE gels using freshly prepared Coomassie blue R250. Tricine gels were prepared as described (Ausubel et al., 1987). Purified C. elegans recombinant NED-8 was subjected to six cycles of Edman degradation (Nucleic Acid and Protein Service Unit, University of British Columbia) and yielded the expected sequence MLIKVK.

**Cloning, Expression, and Purification of C. elegans UBC-12**

A C. elegans cDNA clone, yk486f10, was used as a PCR template for cloning of C. elegans ubc-12. Comparison of the sequence of yk486f10 to the corresponding gene, R09B3.4, showed that the cDNA was missing the first 17 nucleotides of the coding sequence. The complete sequence was obtained by PCR using synthetic oligonucleotides (5’-ccagttgcccagctgtctcttcaacaaagctaaagccagcacaac and 5’-cccaaggccctggaactataaatccagaagtattccc) under the same conditions as given above. The resulting PCR fragment was subcloned into the pRSET A vector and the sequence was confirmed. Recombinant protein was produced in E. coli BL21 (DE3) cells as outlined above. The cell pellet from a 500-ml culture was resuspended in 25 ml of 50 mM phosphate buffer, pH 8, 300 mM NaCl, 10 mM imidazole, and two protease inhibitor tablets (Complete, Mini; Boehringer Mannheim). The mixture was sonicated as described above and the sonicate was then centrifuged at 10,000g for 10 min at 5°C. After clearing, the supernatant was transferred to a 50-ml Erlenmeyer flask. Nickel-charged resin was added, using 0.5 ml of a 50% slurry of Ni-NTA agarose (Qiagen, Inc.) equilibrated in the sonication buffer, and the solution was stirred at 5°C for 30 min. The resin was pelleted (1000g for 30 s) in a conical 50-ml tube and washed twice with 5 ml of buffer B (50 mM phosphate buffer, pH 8, 300 mM NaCl, 20 mM imidazole). The final pellet was resuspended in 2 ml of buffer B and loaded into a 0.7-cm-diameter column and washed with a further 10 ml of buffer B. The bound UBC-12 was eluted in four 0.25-ml fractions of 50 mM phosphate buffer, pH 8, 300 mM NaCl, 250 mM imidazole. The resulting protein was essentially 100% pure and fraction 2 had a concentration of 3 mg/ml.

**Antibody Production**

Rabbit anti-NED-8 antibodies were generated by immunization with recombinant wild-type C. elegans NED-8. Rabbits were immunized with 400 µg of purified NED-8 in Freund’s complete adjuvant and boosted at 2- to 3-week intervals with similar amounts of protein in Freund’s incomplete adjuvant. Antibodies were precipitated from serum with 45% saturated ammonium sulfate and dialyzed against 10 mM Tris-HCl, pH 8.0. Antibodies were affinity purified on an Affi-Gel 10 column (Bio-Rad) to which was coupled 3 mg of purified recombinant wild-type NED-8, as follows: The NED-8 was coupled to 3.0 ml of Affi-Gel 10 in 50 mM NaHCO₃ with gentle mixing overnight at 4°C (as per manufacturer’s instructions). Unreacted active ester sites were quenched by incubation of the resin with 0.1 M ethanolamine hydrochloride, pH 8.0, overnight at 4°C. Rabbit antiserum was adsorbed to the NED-8-Affi-Gel column in 0.1 M sodium phosphate, pH 7.6, and the resin was washed with phosphate buffer containing 0.5 M NaCl. After a final wash with phosphate buffer, bound antibodies were eluted successively with 0.1 M glycine, pH 2.5, and 0.1 M triethylamine, pH 11.5, the eluate being collected into 1/10 volume of 1 M Tris-HCl, pH 8.0. The acid and base eluates were combined, concentrated (Millipore Ultra-Free cartrige, 10-kDa molecular weight cut-off) and dialyzed against 50 mM NaHCO₃.

**Western Blot Analysis**

Proteins were electrophoretically transferred after gel electrophoresis to Immobilon-P (Amersham). The Western blots were blocked for 1 h in 10% nonfat powdered milk in TBS-T (20 mM Tris, pH 7.6, 0.14 M NaCl, 0.05% Tween-20), given three 5-min rinses in TBS-T, and exposed to primary antibody in TBS-T for 1 h. The blots were then given three 5-min rinses in TBS-T and exposed to the appropriate horseradish peroxidase-labeled secondary antibody for 1 h. Following these 3 min rinses in TBS-T, antibody conjugate complexes were visualized by enhanced chemiluminescence (Amersham).

**Northern Blot Analysis**

Northern blots were performed using standard techniques (Sambrook et al., 1989), using 20 µg of total RNA per lane. Northern blots were reprobed after stripping in a solution of 1% SDS, 40 mM Tris, pH 7.6, and 0.1 × SSPE. Message levels were normalized by reference to the level of actin message, itself quantified by hybridization to a 3050-bp EcoRI fragment from the actin1 gene that hybridizes to all four of the C. elegans actin gene transcripts (Krause and Hirsh, 1986). After hybridization and washing, blots were exposed to a phosphor screen and subsequently scanned using a PhosphorImager SI (Molecular Dynamics). Data were analyzed using the IPLab Gel densitometry program (Signal Analytics Corp.) and the averaged pixel densities for each band were compared to the levels of the actin message measured on the same blot, then scaled to give the level in the embryo a value of 100.

**Ubiquitination Assays**

We tested the ability of C. elegans UBC-12 to form a thiol ester bond with either ubiquitin or NED-8 using a modification of published procedures (Hodgins et al., 1996). The ubiquitin and NED-8 activating enzymes were provided by extract prepared from a mixed culture of C. elegans. Live nematodes were washed off plates in a lysis buffer consisting of 50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM DTT, and one protease inhibitor tablet per 10 ml of solution. About 0.25 ml of nematodes (concentrated by a brief centrifugation at 1000g) was suspended in 1.5 ml of the lysis buffer and subjected to 100 strokes in a stainless steel Dounce homogenizer. The homogenate was cleared at 10,000g for 10 min and the supernatant was flash frozen in aliquots and stored at −80°C.
Each 40-μl assay contained 10 mM Hepes, pH 7.5, 5 mM MgCl₂, 40 mM NaCl, 0.1 mM DTT, 1 mM ATP, 2 units of inorganic pyrophosphatase, 4.5 mg/ml phosphocreatine, 14 μg/ml creatine kinase, and 25 μg of C. elegans extract. The latter is required as a source of NED-8 activating enzyme. To this was added 12 μg of purified C. elegans NED-8 or 12 μg of bovine ubiquitin (Sigma) along with 10 μg of either recombinant C. elegans UBC-12 or C. elegans UBC-11, the latter prepared as previously described (Leggett and Candido, 1997). Assays were performed at room temperature for 30 min and the sample was then split into two 20-μl aliquots to which was added SDS sample buffer with or without 2-mercaptoethanol to a final concentration of 360 μM. Proteins were separated by SDS–PAGE (15% (w/v) gel) and transferred to Immobilon-P. The thiol ester-bound ubiquitin was detected by a polyclonal antibody to ubiquitin (a gift from Dr. James Davie) which also detects NED-8.

**Double-Stranded RNA Interference Assays**

Double-stranded RNA was prepared by the method of Fire et al. (1998). Plasmid DNA was linearized with appropriate enzymes and RNA was prepared using T3 and T7 RNA polymerases in separate reactions. Each reaction included roughly 1 μg of linearized plasmid, 1× TSC buffer (40 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 2 mM spermidine, and 10 mM NaCl), 0.5 μM each of the four ribonucleotides, 10 mM DTT, 1 μl (40 U) of RNasin (Promega), and 1 μl (20 U) of T3 or T7 RNA polymerase (Promega). After 90 min at 37°C, 1 μl of each reaction was removed for gel analysis and the remainder was combined and added to 380 μl of a stop mix consisting of 1 M ammonium acetate, 10 mM EDTA, 0.2% SDS, and 1 μl of 20 mg/ml glycogen. This mixture was extracted once with phenol:chloroform (1:1) and once with chloroform. The aqueous phase was incubated at 68°C for 10 min, followed by 37°C for 30 min to anneal the two RNA strands. Double-stranded RNA was precipitated by adding 1 ml of ethanol and centrifuging at 12,000g for 10 min. The pellet was washed with 1 ml of ethanol, air dried, and resuspended in 10 μl of diethylpyrocarbonate-treated distilled water. For shorter dsRNA fragments (fewer than 300 bases), three separate dsRNA preparations were pelleted and combined in 15 μl of diethylpyrocarbonate-treated distilled water. This higher concentration was required since initial tests showed that when shorter RNA fragments were used, the frequency of the affected progeny was reduced.

Double-stranded RNA was microinjected into the gonad syncytia of either N2 hermaphrodites or hermaphrodites of a nonintegrated transgenic roller strain carrying the selectable marker rol-6 (Kramer et al., 1990) and a fusion of the body wall myosin gene unc-54 with the green fluorescent protein gene (pPD93.48; a gift from A. Fire). Injected individuals were allowed to recover on plates with bacterial food for approximately 4 h in order to purge any already fertilized embryos, then transferred to fresh plates and allowed to lay eggs for roughly 17 h (brood A). They were then transferred to fresh plates and allowed to lay eggs for an additional 8 h (brood B). Among other differences, the two broods might be expected to show different maternal effects since the production of targeted protein will be inhibited in the injected hermaphrodite as well as in its progeny (Fire et al., 1998).

We tested the RNAi technique using a nonintegrated transgenic line carrying a plasmid encoding a fusion of green fluorescent protein (GFP) to the promoter of a myosin heavy chain gene (unc-54). With this strain, injection of dsRNA corresponding to the GFP coding sequence provided a simple control of injection success and effectiveness of the RNAi technique. Individuals injected with GFP dsRNA produced progeny which lacked GFP in all body muscle except the vulval muscle cells (not shown), confirming a previous report suggesting that the effectiveness of RNAi diminishes in cells which differentiate late in development (Fire et al., 1998). Fully 87% of the roller progeny in brood A and 100% of those in brood B were affected in these experiments.

**RESULTS**

**Characterization of C. elegans NED-8**

The NED-8 protein of C. elegans is encoded on chromosome I, cosmid sequence F45H11 (the NCBi designation for the protein is F45H11.2), and consists of 77 amino acids with a terminal phenylalanyl residue. C. elegans NED-8 has a predicted molecular mass of 8482 and a predicted pl of 6.58. The ned-8 gene is oppositely transcribed from another gene encoding a putative protein (F45H11.3) of 743 amino acids which lies only 954 bp away (Fig. 1A). F45H11.3 has no known protein domains as determined by the Pfam database (Sonhammer et al., 1998) and no known related proteins in other organisms. Southern blots of C. elegans genomic DNA digested with EcoRI and Hpal were probed with a ned-8 cDNA and showed that ned-8 was a single-copy gene (results not shown).

A single cDNA encoding C. elegans NED-8 was previously described (McCombie et al., 1992). This clone, T01966, encodes the complete coding region except for the initiator methionine and has a 3' noncoding region of 188 bp. The genomic sequence of ned-8 includes an acceptor splice site 13 bp upstream of the methionine initiator codon, suggesting that the gene is trans-spliced (Krause and Hirsh, 1987). NED-8 is encoded by two exons separated by an intron of 68 bp. This intron differs in position from that found in three of the coding units of the C. elegans ubiquitin gene (ubq-1; Fig. 1C). An alignment of several NED-8-related proteins (Fig. 1B) shows that the amino acid sequence of C. elegans NED-8 is 88% identical to the murine protein (NEDD8), but only 59% identical to the S. cerevisiae homolog, RUB1. C. elegans NED-8 is 60% identical to C. elegans ubiquitin, and its predicted secondary structure is nearly identical to that of ubiquitin (Fig. 1B).

The C. elegans ned-8 coding sequence minus the terminal phenylalanine codon was subcloned into the pRSET expression vector (Invitrogen). The terminal phenylalanine was removed since the active form of the protein likely has a terminal glycyl residue, in concordance with the posttranslational processing of ubiquitin (Ozkaynak et al., 1984). A His6-tagged and a nontagged (native) form were both overproduced in E. coli and found to be soluble. Native NED-8 was purified from E. coli extracts by ammonium sulfate precipitation. NED-8 remained soluble in saturated ammonium sulfate and was essentially pure after this single-step procedure (Fig. 2). Where needed, impurities were removed by passing the dialyzed and concentrated ammonium sulfate fraction over a Bio-Gel P10 gel filtration column. The purified protein was...
visualized on Tris/Tricine gels on which it ran with an apparent molecular mass of 6 kDa, identical to the apparent size of bovine ubiquitin on the same gels. As NED-8 does not contain any tryptophan and only a single tyrosyl residue, it could not be detected by UV absorbance. The protein also reacted poorly with Coomassie blue stain, perhaps due to the rarity of basic residues in its sequence.

Polyclonal antibodies were raised against recombinant nontagged NED-8 and affinity purified by binding to recombinant NED-8 covalently linked to Affi-Gel 10. The resulting antibody was highly specific for NED-8 and did not react appreciably with bovine ubiquitin (Fig. 3A) or with *S. cerevisiae* extracts (data not shown), showing it did not cross react with yeast RUB1 or yeast ubiquitin.
C. elegans embryo extracts contained two prominent anti-NED-8 reactive bands. One of these was 6 kDa, and the other 90 kDa (Fig. 3B), with several fainter bands also apparent. A 180-kDa species was seen in some embryo extracts, which may be an artifactual dimer of the 90-kDa protein. Extracts made from C. briggsae, a closely related nematode species, also contained two reactive bands of similar sizes (Fig. 3C). The NED-8 antibody also detected two major bands of 6 and 55 kDa in HeLa cell extracts (Fig. 3D). The specificity of the NED-8 antibody was checked by preincubating the antibody with an excess of NED-8. Such pretreated antibody did not react with NED-8 or the 90-kDa conjugate (data not shown).

Immunoprecipitation experiments showed that the NED-8 antibody was able to isolate NED-8 from C. elegans embryo extracts but failed to immunoprecipitate the 90-kDa protein (results not shown). This suggested that the NED-8/90-kDa protein may be part of a complex in which NED-8 is buried internally and not accessible to antibody binding. This interpretation was reinforced by results obtained from immunoaffinity studies using NED-8 antibody bound to Affi-Gel 10 resin. When C. elegans embryo extracts were passed over such a column, none of the NED-8/90-kDa protein was bound, further suggesting that the NED-8 moiety was not accessible to the antibody.

Immunoblotting showed that NED-8 was present throughout C. elegans development (Fig. 4A). In contrast, the 90-kDa protein was very prominent in embryos, but was nearly absent in the first three larval stages and the dauer larvae. The 90-kDa protein reappeared in fourth stage larvae and was also present in adult nematodes. Several other reactive protein species were visible, especially in the larval stages.

Identification and Characterization of the NED-8 Conjugating Enzyme in C. elegans

We next attempted to determine which of the C. elegans ubc genes was responsible for NED-8 conjugation. Twenty predicted C. elegans UBC proteins and 13 yeast UBC proteins were analyzed using a Web-based phylogenetic tree program (www2.ebi.ac.uk/clustalw). The results (not shown) suggested that yeast UBC12, which mediates RUB1 conjugation (Liakopoulos et al., 1998), was most similar to the C. elegans protein R09B3.4 encoded on chromosome I. Alignment of R09B3.4 to human and yeast UBC12 proteins (Fig. 5) showed that R09B3.4 was 28% identical in amino acid sequence to yeast UBC12 and 41% identical to human UBC12. The protein corresponding to R09B3.4 was expressed and purified as outlined under Materials and Methods and then tested in an in vitro assay for its ability to form a thiol ester bond with C. elegans NED-8. As a control in these experiments we used another C. elegans ubiquitin conjugating enzyme, UBC-1 (Leggett et al., 1995). Since this protein is itself covalently modified by autoubiquitination at lysine-162 (Leggett and Candido, 1997), we used a carboxyl-terminal deleted version (UBC-1 \( \Delta \)152) which lacks this modification site, to simplify the interpretation of the results. UBC-1 \( \Delta \)152 is able to form a thiol ester bond with ubiquitin at its active-site cysteine (Fig. 6A, lanes 1 and 2), but is unable to bond with NED-8 (Fig. 6B, lanes 1 and 2). In contrast, R09B3.4 was able to form a thiol ester bond with NED-8 (Fig. 6B, lanes 3 and 4) but not with ubiquitin (Fig. 6A, lanes 3 and 4). Since the activating enzymes for NED-8 and ubiquitin were provided by a C.

**FIG. 4.** Developmental profile of NED-8 conjugates in C. elegans. The Western blot contains 10 \( \mu \)g of total protein extract from each stage of C. elegans development and was developed using anti-NED-8 pAb. (E, embryo; L1 to L4, larval stages 1 to 4; YA, young adults (not gravid); A, gravid adults; and D, dauer larvae.)

**FIG. 5.** Comparison of *S. cerevisiae* UBC12 (Accession No. X99442), C. elegans UBC-12 (R09B3.4, Accession No. Z81108), and human UBC12 (Accession No. AF075599). Dark shading indicates a position where two or more sequences have identical amino acids and light shading denotes conservative substitutions.
**Identification of the NED-8 Activating Enzymes in C. elegans**

In *S. cerevisiae* two proteins, *ULA1* and *UBA3*, form a heterodimeric RUB1 activating enzyme (Liakopoulos et al., 1998) and orthologs of these two proteins occur in the human genome (Gong and Yeh, 1999) and in *C. elegans*. The two *C. elegans* genes encoding homologs of *ULA1* (C26E6.8) and *UBA3* (F11H8.1) are both located on chromosome III, approximately 10 map units apart. The amino acid sequence of *C. elegans* ULA-1 is 37% identical to its human homolog, while *C. elegans* UBA-3 is 51% identical to its human homolog. An alignment of the *C. elegans* ULA-1 and UBA-3 proteins with a *C. elegans* homolog of the yeast activating enzyme UBA1 indicated that both the ATP binding domain and the active site were located in the UBA-3 component (not shown).

**Developmental Regulation of NED-8 Activating and Conjugating Enzymes**

A labeled probe made from the *ned-8* cDNA detected a message of approximately 500 nucleotides in all life stages of *C. elegans* including the embryo, the four larval stages, the adult, and the dauer larva (results not shown). We tested the possibility that the levels of NED-8 conjugates might be regulated by changes in the level of expression of the genes encoding the NED-8 activating and conjugating enzymes. Probes for *C. elegans* ned-8, ubc-12, and uba-3 showed, however, that message for each of these genes was present in all developmental stages with the level of the *ned-8* message being somewhat higher in the L4 stage than at other times during development. (Fig. 7).
three components of the NED-8 conjugation system was used in RNAi experiments. In the case of ned-8, the coding sequence is 63% identical to the coding sequence of C. elegans ubq-1 (there are 11 coding units of ubiquitin in ubq-1), and it was possible that a coding region ned-8 dsRNA might target other ubiquitin or ubiquitin-related genes. Therefore, a dsRNA fragment corresponding only to the 3' untranslated 188 bases of the ned-8 message (Fig. 1D) was tested in three separate RNAi experiments in which 25, 23, or 23 hermaphrodites were injected. A low frequency of embryonic and larval arrest was seen in the progeny of these treated nematodes (Table 1). Interestingly, a significant proportion of the surviving larvae developed a large vulval protrusion during the L4 larval stage (Fig. 8A). When these animals molted to the adult stage, they generally burst at the vulva (Fig. 8B) and died. Many of the adults which did not burst were egg-laying defective and their larvae hatched internally. In a typical experiment in which 23 hermaphrodites were injected, 1392 larvae were produced, with a hatching frequency of 89% for brood A and 77% for brood B (Table 1). The hatching success was therefore somewhat greater in brood A than in brood B, perhaps due to maternally inherited NED-8. The first brood had a larger proportion of individuals with a vulval evisceration or which burst at the L4 to adult molt. Again, maternally inherited NED-8 might have allowed a greater proportion of the first brood to survive to later stages at which other abnormalities appear. In one ned-8 RNAi experiment, six male progeny were observed and all of these had abnormal tails, with reduced size and number of rays (Fig. 8C).

The progeny of hermaphrodites injected with the ned-8-specific dsRNA also displayed another striking phenotype, abnormal morphology of the lateral alae. Alae are produced on the adult cuticle as an extension of the lateral seam cells (White, 1988) and consist of three raised ridges running the length of the nematode on each side of the body (Fig. 8D). Individuals affected by ned-8 dsRNA had patchy interruptions in the alae in which the ridges were absent or replaced by an irregular pattern of small raised spots (Fig. 8E). Alae frequently diverged around a central patch of cuticle. The latter “traffic islands” were not raised above the cuticle surface and had no obvious surface features at 1000× magnification (Fig. 8F). Portions of the alae on some individuals diverged at right angles to the body axis. Abnormalities in alae were common on animals which had burst at the vulva during the L4 to adult molt, but were also seen on adults which had not burst. Generally, however, individuals with abnormal alae also exhibited other damage, such as vulval protrusion or an egg-laying defect. Alae did not form prematurely on RNAi-treated nematodes: L2, L3, and L4 stage progeny were found to lack alae, as is the case in normal development.

The putative phenotype of a C. elegans ubc-12 knockout was determined by RNAi using dsRNA prepared from a cDNA (yk486f10) corresponding to the ubc-12 gene. This incomplete cDNA includes 526 bp of coding sequence and 59 bp of 3' noncoding sequence. The phenotype of the progeny of nematodes injected with yk486f10 dsRNA included embryonic arrest, vulval evisceration at the L4 stage (Fig. 9A), and bursting at the vulva during the L4 to adult molt. The second brood suffered a much higher frequency of embryonic arrest than the first brood (55% compared to 6%), suggesting a maternal effect rescue in the first brood for this aspect of the phenotype. Since the penetrance of UBC-12 RNAi-induced abnormalities was somewhat higher than that seen with NED-8 RNAi, we used UBC-12 RNAi to examine the stage of embryonic arrest. The embryonic arrest was at the comma to tadpole stage (Fig. 9C). The arrested embryos contained a few gut granules and limited muscle cell movement.

Vulval precursor cells were examined on L2 and L3 stage RNAi-treated individuals. Cell numbers were normal to the L3 stage, but in the L4 stage gross abnormalities in morphology began to appear and the cell numbers could not be readily determined. Specimens that survived to the adult stage displayed severe abnormalities in the structure of the alae (Fig. 9B). As was the case with ned-8 RNAi, the alae were patchy, branched, or diverged around a central space which in this case sometimes contained irregular granular deposits. L3 and L4 stage larvae had normal appearing seam cells, but the seam cell nuclei were indistinct on the adults and granular material was found in the hypodermis underlying abnormal, patchy alae.

Another phenotype induced by ubc-12 RNAi was a truncation of the posterior region of the body. This was noted as a rare occurrence in the second brood, but was also seen in the second generation of progeny after injection. Varying degrees of this abnormality were seen in different spec-

<table>
<thead>
<tr>
<th>Brood</th>
<th>ned-8 RNAi</th>
<th>ubc-12 RNAi</th>
<th>ula-1 RNAi</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Hatching frequency (%)</td>
<td>89</td>
<td>77</td>
<td>93</td>
</tr>
<tr>
<td>Bursting at L4 to adult molt and protruding vulva (%)</td>
<td>18</td>
<td>10</td>
<td>20</td>
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<tr>
<td>Internal hatching of embryos (%)</td>
<td>5</td>
<td>1.5</td>
<td>0</td>
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</tbody>
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Note. nd, not determined.
FIG. 8. Phenotypes of ned-8 RNAi-treated nematodes. (A) Everted vulva on an L4 stage hermaphrodite. (B) A burst adult after the L4 to adult molt. (C) Abnormal tail fan and rays in a male affected by ned-8 dsRNA. (D) Normal alae on the cuticle of an adult, untreated hermaphrodite. (E) Abnormal, granular alae on an adult hermaphrodite. (F) Abnormal alae showing “traffic islands.” All individuals except (D) are progeny of hermaphrodites injected with the ned-8 3’ noncoding sequence dsRNA. The scale bars represent 25 μm with A and C at the same scale and D, E, and F at the same scale.
mens, with the minimal effect being a hooked tail (Fig. 9D). Although these individuals developed to the adult stage, they displayed a disorganized gonad and produced no progeny. The alae on these individuals were highly branched in the tail region, but normal over the anterior part of the animal. Hypodermal cells were densely packed in the tail region, with normal numbers of seam cells in the anterior of the animal.

The consistency of the phenotypes of both ned-8 and ubc-12 RNAi experiments prompted us to also examine the effects of RNA interference with ula-1, a component of the NED-8 activating enzyme. A full-length cDNA sequence (yk433e12) corresponding to the C. elegans ula-1 gene was used to prepare dsRNA, which was injected into either N2 or roller unc-54-GFP C. elegans in separate experiments. The most obvious phenotype of the progeny of ula1-dsRNA treated nematodes was bursting at the vulva during the L4 to adult molt (Figs. 9E and 9F). This was particularly

**FIG. 9.** Phenotypes of ubc-12 and ula-1 RNAi-treated nematodes. The ubc-12 RNAi phenotype includes: (A) everted vulva and abnormal alae (arrow) on an adult, (B) abnormal alae on an adult cuticle, (C) embryonic arrest at the tadpole stage, and (D) hooked tail on an L1 larva of the second postinjection generation. The phenotype of ula-1 RNAi includes: (E) everted vulva and abnormal alae (arrow) on an adult and (F) bursting at the vulva after the L4 to adult molt. The scale bar represents 25 μm with A, B, D, E, and F at the same scale.
striking in brood B in which 31% of the progeny burst at the L4 to adult molt (Table 1). Vulval protrusion and an egg-laying defect also occurred in the progeny of ula-1 dsRNA-treated nematodes. However, unlike the case with ned-8 and ubc-12 dsRNA treatment, embryonic arrest was not observed. In addition, the progeny of ula1-RNAi-treated nematodes showed a low frequency of abnormalities in the alae (Fig. 9E) which were strikingly similar to the alae abnormalities produced by ubc-12 and ned-8 RNAi (compare Figs. 9A and 9E).

DISCUSSION

It is now well established that NEDD8 is conjugated to the cullins, a family of six scaffold proteins, at least two of which are known to participate in multiprotein complexes involved in recognition and ubiquitylation of specific target proteins. The first cullin shown to be modified by NEDD8/RUB1 was S. cerevisiae CDC53 (Liakopoulos et al., 1998). Subsequent studies have shown that human cullin 1 and cullin 2 are also neddylated (Liakopoulos et al., 1999; Wada et al., 1999a) as are the other four mammalian cullins (Hori et al., 1999). NEDD-8 is conjugated to a lysyl residue within the sequence VRIMK, which is conserved in almost all known cullins including those of C. elegans (del Pozo and Estelle, 1999; Wada et al., 1999b). If NEDD8 modification of the cullins is essential for cullin function, then the phenotype produced by RNA interference with the neddylation system should be related to the phenotypes of cullin mutants. Such mutants have been described for both C. elegans CUL-1 and CUL-2. CUL-1 mutants display embryonic arrest with hyperplasia in all tissues (Kipreos et al., 1996). A CUL-2 knockout results in embryonic arrest with CUL-2 required both for the G1-to-S phase transition and for chromosome condensation (Feng et al., 1999). C. elegans RNA interference assays using double-stranded RNA encoding NED-8, the NED-8 activating enzyme component ULA-1, or the NED-8 conjugating enzyme UBC-12 all resulted in an embryonic arrest phenotype. Moreover, there was a striking similarity between the tissue distribution of CUL-2 in C. elegans and the tissues that were affected by ned-8 RNA interference. While CUL-2 protein levels generally drop in the larval stages, relatively high levels of CUL-2 are maintained in the seam cells throughout development and in the vulval and somatic gonad cells in the late L3 and L4 stages. The seam cells are positioned along both lateral lines of the nematode. In the adult, the seam cells fuse into two seam cell syncytia and raised cuticular ridges called alae form in the cuticle above the syncytia (White, 1988). One of the most striking defects produced by ned-8 RNA interference was failure to form normal alae. The alae were disrupted, with irregular patches interrupting the usual continuous ridges in the cuticle above the seam cells. This effect was most pronounced when UBC-12 was knocked out by RNA interference, eliminating the possibility that maternally inherited NED-8 could be used for cullin modification.

A second, and highly penetrant, effect of ned-8 RNA interference was a defect in vulval formation that resulted in an everted vulva in the L4 stage and either bursting of the body in the vulval region during the final molt or an egg-laying defect in the adult. The terminal differentiation of the vulva in C. elegans involves a complex series of cell fusion events and muscle attachments (Sharma-Kishore et al., 1999). The bursting of ned-8 RNAi-treated individuals suggests that the fusion events or cell attachments may not have occurred, resulting in a structural weakness in the vulva that was unable to withstand the turgor pressure of the body. Since the terminal differentiation of both the seam cells and the vulva involve cell fusion events, the abnormalities noted in ned-8 RNAi-treated nematodes suggests that the cullins may play an essential role in cell fusion processes, as has been demonstrated in myoblast cells (Gardrat et al., 1999) in which the inhibition of the proteasome or of ubiquitin ligases represses cell fusion. The similarities between the tissues affected by the ned-8 RNAi phenotype and the tissue distribution of CUL-2 suggest that CUL-2 may be a major target for NED-8 modification in C. elegans. This conclusion is reinforced by the observation that the message levels of five of the cullins drop in the larval stages, as does the level of CUL-2 protein (Feng et al., 1999; Kipreos et al., 1996). This also resembles the developmental profile of the level of the NED-8 conjugate in C. elegans, a profile which could not be explained by corresponding changes in the availability of the conjugating or activating enzymes for NED-8, since the message levels for these enzymes were fairly constant throughout development.

The observation that the NED-8 conjugating system in C. elegans is essential for normal development is consistent with the phenotypes of mutations in components of the neddylation system in other organisms. Deletion of the gene ENR2 (ULA1), part of the heterodimeric RUB1 activating enzyme, is lethal in S. cerevisiae strains expressing temperature-sensitive alleles of cdc34, a target for ubiquitylation by the SCFcdc4 complex (Lammer et al., 1998). Mutations in the Arabidopsis AXR1 gene, an ortholog of ENR2, affect the response to the phytohormone auxin (del Pozo et al., 1998) with resulting abnormalities in root gravitropism and lateral root formation (Hobbie and Estelle, 1995). It has recently been shown that an Arabidopsis ortholog of cullin 1 (AtCUL1) is modified by RUB1 and that AtCUL1 is part of an SCF complex that presumably targets negative regulators of the auxin response for degradation (del Pozo and Estelle, 1999). Mutations in the S. pombe rad31 gene, another ortholog of ENR2, produce sensitivity to UV and growth defects at elevated temperature (Shayeeghi et al., 1997).

The effects of ned-8 RNA interference on the development of the vulva and lateral seam cells are similar to the phenotypes of several well-characterized C. elegans mutants. For example, terminal differentiation of the lateral
seam cells in C. elegans requires the presence of LIN-29 protein, one of the heterochronic gene products (Liu et al., 1995; Rougvie and Ambros, 1995). The activity of LIN-29, a transcription factor involved in the regulation of adult-specific collagen genes, is inhibited in the larval stages by the products of other genes, notably lin-28. In the absence of LIN-28, the lateral alae develop prematurely, in larvae. In the absence of LIN-29, no alae form in the adult. Animals lacking LIN-29 also show abnormal vulval development leading to a protruding and nonfunctional vulva (Bettinger et al., 1997). Abnormalities in the alae and vulva may be related since some seam cells contribute to vulval morphogenesis (Bettinger et al., 1997; Newman et al., 1996). LIN-29 is also essential for male tail development (Euling et al., 1999), and seam cells in the tail region contribute to the tail structure. Although only a few males were examined in the present study, ned-8 RNA interference also affected the formation of the rays, a product of seam cell differentiation in the male. It is likely that ubiquitin-mediated protein degradation plays an important role during terminal differentiation of the seam cells, the vulva, and the male tail. Since ned-8 RNA interference affects the structure of the alae rather than the timing of their development, the levels of LIN-29 are not likely affected by ned-8 RNAi. More likely, the products of genes regulated by LIN-29 may be affected, or there may be other proteins which must be degraded before terminal differentiation can occur. For example, the collagen gene col-17 is repressed in the adult while col-19 is activated (Liu et al., 1995). The complete removal of larval-specific collagens or other larval-specific proteins may require ubiquitin-mediated degradation directed by a ubiquitin ligase complex containing NED-8 modified cullins.

Two main hypotheses have been proposed for the role of neddylation of the cullins. First, NEDD8 may target the modified protein to a cell compartment analogous to the nuclear compartmentalization seen with proteins modified by SUMO, another ubiquitin-like protein (Matunis et al., 1998). Consistent with this hypothesis, a large fraction of NEDD8 modified protein in mammalian cells is localized to the nucleus (Kamitani et al., 1997), and the Arabidopsis AXR1 protein is similarly partitioned (del Pozo et al., 1998). A second hypothesis is that NEDD8 modification affects the ability of the modified cullin to interact with other members of the SCF complex or with target proteins of the SCF. In S. cerevisiae, the phenotypes of alleles of the SCF components CDC53, CDC34, CDC4, and SKP1 are more severe when expressed in cells that are nullizygous for RUB1, suggesting a role for RUB1 modification in the assembly of the functional SCF (Lammer et al., 1998). Immunoprecipitation studies have shown a conformational change in human CUL2 after NEDD8 modification (Wada et al., 1999a) which disclosed antigenic determinants at the N terminus of the molecule. Such conformational changes might be critical for SCF assembly or for substrate recognition by the mature assembly.

The present work demonstrates that disruption of the neddylation system has pleiotropic effects on development. This should perhaps be expected on the basis of known characteristics of the SCF-type ubiquitin ligases. For example, the VCB-CUL2 complex is known to associate with the BC-box protein, VHL, and likely with others (for a recent review see Tyers and Rottapel, 1999). The different associated proteins allow for a larger repertoire of substrates for the SCF complexes and the variety of phenotypes noted here may reflect a lack of degradation of a variety of substrate proteins. In C. elegans, maternal effect rescue of ned-8 RNA interference allows some treated individuals to develop past the embryonic stage, unmasking other functions of the SCF complexes and revealing the full range of developmental pathways in which NED-8 modified cullins play a role. Alternately, NED-8 may modify other proteins unrelated to the cullins. One of the puzzling aspects of the ubc-12 RNAi phenotype, for example, was an abnormality in the development of the hermaphrodite tail. There is currently no clear link between this phenotype and the expression pattern of CUL-2, but the defect, which overtly resembles that produced by mutations in the HOX gene nob-1 (Van Auken et al., 2000), suggests that ubiquitin-mediated protein degradation is essential in the development of the tail region of early stage C. elegans larvae.

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REFERENCES


Feldman, R. M., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes...


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