The *bli-4* locus of *Caenorhabditis elegans* encodes structurally distinct kex2/subtilisin-like endoproteases essential for early development and adult morphology

Colin Thacker, Ken Peters, Martin Srayko, and Ann M. Rose

Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

Many secreted proteins are excised from inactive proproteins by cleavage at pairs of basic residues. Recent studies have identified several serine endoproteases that catalyze this cleavage in the secretory pathways of yeast and metazoans. These enzymes belong to the kex2/subtilisin-like family of proprotein convertases. In this paper we describe the molecular characterization of the *bli-4* gene from *Caenorhabditis elegans*, which was shown previously by genetic analysis of lethal mutants to be essential for the normal development of this organism. Sequencing of cDNA and genomic clones has revealed that *bli-4* encodes gene products related to the kex2/subtilisin-like family of proprotein convertases. Analysis of *bli-4* cDNAs has predicted four protein products, which we have designated blisterases A,B,C, and D. These protein products share a common amino terminus, but differ at the carboxyl termini, and are most likely produced from alternatively spliced transcripts. We have determined the molecular lesions for three *bli-4* alleles (*h199*, *h1010*, and *q508*) that result in developmental arrest during late embryogenesis. In each case, the molecular lesions are within exons common to all of the BLI-4 isoforms. The original defining allele of *bli-4*, *e937*, is completely viable yet exhibits blistering of the adult cuticle. Molecular analysis of this allele revealed a deletion that removes exon 13, which is unique to blisterase A. No RNA transcript corresponding to exon 13 is detectable in the blistered mutants. These findings suggest that blisterase A is required for the normal function of the adult cuticle. The *bli-4* gene is a complex locus as evidenced by the characterization of mutant strains and RNA transcripts. Furthermore, our data show that functional redundancy may exist among the various BLI-4 isoforms.

[Key Words: *Caenorhabditis elegans*; KEX2; serine endoproteases; blisters; proprotein processing]

Received November 30, 1994; revised version accepted March 8, 1995.

Many biologically active secreted proteins are synthesized as large inactive precursors. Activation occurs during the secretion process by limited endoproteolytic cleavage, most frequently after pairs of basic amino acids such as Lys-Arg or Arg-Arg (for review, see Docherty and Steiner 1982; Thomas et al. 1988). This cleavage occurs by a general mechanism found in all eukaryotes examined to date, and is catalyzed by a group of serine endoproteases called the kex2/subtilisin-like family of proprotein convertases (for review, see Barr 1991; Seidah and Chrétien 1992). Criteria for assigning enzymes to the family include the ability to process precursor proteins at dibasic sites, and sequence conservation of the protease domain with the bacterial subtilisins and the yeast endoprotease Kex2p. The prototype member of this family of convertases, Kex2p, is a membrane-bound Ca^{2+} -dependent serine endoprotease that cleaves the pro- α -maturing factor at dibasic sites (Mizuno et al. 1988; Fuller et al. 1989a; for review, see Fuller et al. 1988). Mamma-

lian members of the family of convertases include PC1/PC3 (Seidah et al. 1991; Smeekens et al. 1991), PC2 (Seidah et al. 1990; Smeekens and Steiner, 1990), PC4 (Nakayama et al. 1992; Seidah et al. 1992), PC5/PC6 (Lusson et al. 1993; Nakagawa et al. 1993), PACE4 (Keifer et al. 1991), and human furin (hFURIN; Roebroek et al. 1986; Fuller et al. 1989b; van den Ouweland et al. 1990; Wise et al. 1990). Defining the biological significance of individual convertases has been stymied by the lack of evidence demonstrating substrate specificity. For example, when removed from their biological context, many of the endoproteases are able to process the same substrates, raising the question whether such a functional redundancy exists among the family members in vivo. Some insight into defining functionality has been provided by examining the expression patterns and localization of the individual convertases. From such analyses it has been shown that substrate specificity can be influenced by both restricting expression to particular tissues

and compartmentalization of the individual enzymes to specific intracellular locations (Seidah and Chrétien 1992).

Restricted expression is exhibited by PC1/PC3 and PC2 to endocrine and neuroendocrine tissues (Seidah et al. 1990, 1991; Smeekens and Steiner 1990; Smeekens et al. 1991), whereas PC4 is restricted to the testis (Nakayama et al. 1992; Seidah et al. 1992). These observations imply a distinct functionality for the three convertases within the regulated secretory pathway. In contrast, both hFURIN and PACE4 are expressed in a broad range of tissues suggesting that these convertases are required for processing activity within the constitutive secretory pathway (Roebroek et al. 1986; Bresnahan et al. 1990; van den Ouweland et al. 1990; Van de Ven et al. 1990; Kiefer et al. 1991). PC5/PC6, like hFURIN and PACE4, exhibits a widespread tissue distribution. However, levels of expression are predominant in intestinal tissue (Lusson et al. 1993; Nakagawa et al. 1993). This may suggest that PC5/PC6 is active in both the constitutive and regulated secretory pathways. Clearly, an added complication in defining substrate specificity arises within cells in which regulated and constitutive pathways are active, as both types of convertase will be produced.

Compartmentalization may also influence the control of proprotein convertase substrate specificity and activity. Individual enzymes are sequestered in separate intracellular compartments. Localization of the individual processing enzymes appears to be a function of the structural differences between each family member. This has been demonstrated clearly for hFURIN, which is concentrated in the *trans*-Golgi network (TGN) (Molloy et al. 1994). hFURIN, like all members of the kex2/subtilisin-like family, is synthesized as an inactive zymogen (Leduc et al. 1992; for review, see Seidah and Chrétien 1992). Exit from the endoplasmic reticulum and transport to the TGN appears to coincide with activation of the catalytic function of the enzyme. Furthermore, the intracellular distribution of hFURIN is dynamic. The enzyme is cycled to and from the TGN by the cell surface within coated vesicles. At least in part, the trafficking signals required for cycling are encoded within the cytoplasmic tail of hFURIN (Molloy et al. 1994).

The identification of kex2/subtilisin-like proprotein convertases in simple animal systems that are amenable to classical and molecular genetic manipulation provides an alternate approach to examine the role of these important enzymes. Recently, genes that encode kex2/subtilisin-like endoproteases have been isolated from *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. Two genes isolated from *Drosophila*, called *Dfur-1* (Roebroek et al. 1992, 1994; also called dKLIP-1; Hayflick et al. 1992), and *Dfur-2* (Roebroek et al. 1992), encode convertases with sequence similarity to hFURIN. Genes isolated from *C. elegans* include CELPC2, which shows sequence similarity to PC2 (Gómez-Saladín et al. 1994), and an additional gene identified by the genome sequencing group that most closely resembles hFURIN (Waterston et al. 1992; C. Thacker

and A.M. Rose, unpubl.). At present, no known mutations within these genes have been reported.

Previously, this laboratory described the genetic analysis of mutants within the *bli-4* gene of *C. elegans*. In this report we present the characterization of the *bli-4* gene and show that it encodes serine endoproteases that belong to the kex2/subtilisin-like proprotein convertase family. *bli-4* was first identified by a recessive mutant that results in the formation of fluid-filled separations, or blisters, of the adult cuticle layers (Brenner 1974). Characterization of the *e937* mutation revealed it to be incompletely penetrant; 85%–90% of *e937* homozygotes exhibit the blistered phenotype (Brenner 1974; Peters et al. 1991). Subsequently, 13 additional recessive mutants were isolated, all of which are lethal, arresting development in late embryogenesis (Rose and Baillie 1980; Howell et al. 1987; Peters et al. 1991; C. Thacker, M. Srayko, and A.M. Rose, unpubl.). The associated lethality indicates an essential role for *bli-4* in embryogenesis in addition to its role in the production or maintenance of the adult cuticle. Here we show that the *bli-4* gene encodes at least four proprotein convertases that arise by alternative splicing at the 3' end, generating enzymes with different structural properties. The four gene products share the protease domain yet differ at the carboxyl termini. Alternative splicing generates isoforms with structural similarities to each of the mammalian proprotein convertases that participate in both the regulated and constitutive secretory pathways. Molecular lesions responsible for three lethal mutants were identified within coding sequences common to all of the BLI-4 isoforms. Removal of the unique 3' exon of one isoform results in the blistered phenotype. Our results also suggest that incomplete penetrance of blistering exhibited by the *e937* mutation may result from functional redundancy among the various *bli-4* gene products. This is the first identification of mutants in a kex2/subtilisin-like gene in a multicellular organism, and the first direct evidence that a member of this gene family is essential for development.

Results

Mutants display both a viable cuticular defect and lethality

Previous genetic analysis in our laboratory has demonstrated that *bli-4* is a complex locus (Peters et al. 1991). Mutations within the gene have been categorized into three classes depending on mutant phenotype and intra-complementation analysis (Table 1). The first mutant class is represented by the recessive allele *e937*, which originally defined the locus (Brenner 1974), and exhibits blistering of the adult cuticle but is otherwise completely viable (Fig. 1B). The *e937* mutation shows incomplete penetrance such that ~90% of homozygous animals display the blistered phenotype. Currently, there are 13 known recessive alleles of *bli-4* that result in lethality. These alleles exhibit a complex pattern of intra-genic complementation and have been grouped into two

Table 1. Intracomplementation between *bli-4* alleles

a/b ^a	Class I ^{a,b}	Class II ^a	Class III ^a
Class I	Bli (90%)	—	—
Class II	Bli (100%)	arrest threefold	—
Class III	wild type	arrest threefold	arrest threefold/ L1 larvae

^aClass I mutant is represented by *e937*. Class II mutants are represented by *h1010* and *q508*. The class III mutant is represented by the single allele *s90*.

^bPercentage of blistered animals. Approximately 90% of *e937* homozygotes exhibit the blistered phenotype. The blistered cuticle phenotype is fully penetrant in the *e937*/class II lethal mutant heteroallelic combination.

separate classes based on their complementation with each other and with *e937* (Table 1). Twelve lethal alleles that do not complement *e937* represent class II mutants and arrest development in late embryogenesis during elongation of the embryo. An example of the terminal phenotype of a class II mutant is shown in Figure 1D. Arrested animals appear fully differentiated yet are unable to complete elongation (Fig. 1, cf. C and D). All class II mutants examined become vacuolated, particularly in the head region (Fig. 1D). A single recessive lethal allele, *s90*, complements *e937* and represents a third *bli-4* mutant class (class III). Normally, one would interpret the complementation of *e937* by the class III allele to mean that these mutations are in two separate genes. However, *s90* fails to complement any of the class II lethal alleles, suggesting that the mutation does reside in the same gene. Most animals homozygous for the *s90* mutation arrest at the same stage of development and are indistinguishable in phenotype from the class II lethal mutants. However, ~30% of the animals hatch and later arrest as early L1 larvae with a distinctive “dumpy” appearance (Fig. 1F). Apart from the obvious morphological defect, the larvae respond to touch and pharyngeal pumping is observed, albeit at a very reduced rate. These dumpy animals persist for several days before dissolution. The mutant phenotypes observed for both the class II and III lethal alleles are similar to those seen for cuticle-defective mutants that elongate normally, but subsequently retract in length (Priess and Hirsh 1986). Isolation of lethal mutants in *bli-4* implies an essential function for the gene, in contrast to the cuticle defect associated with the original *e937* mutation. The interesting genetics associated with the various mutants and the intriguing incomplete penetrance of the blistered mutant prompted us to characterize the gene further.

The *bli-4* locus is situated in cosmid K04F10

To clone the *bli-4* gene, we aligned the genetic map position with the physical map as described in Materials and methods (Fig. 2). Using the cosmid K04F10, we were able to identify DNA rearrangements associated with two *bli-4* mutants, *h1010* and *e937*. *h1010* was isolated in a precomplementation screen for *bli-4* mutations in a

mut-6 background (Peters et al. 1991). *mut-6* mobilizes the transposable element Tc1 (Mori et al. 1988); therefore, *h1010* was presumed to contain a transposon insertion in *bli-4*. We identified the Tc1 insertion site in a 1.3-kb *EcoRI* fragment (Fig. 3A; see below). Analysis of genomic DNA isolated from *e937* homozygotes using K04F10 as probe revealed a deletion of 3.5 kb (Fig. 3A). Thus, two DNA rearrangements associated with two independent *bli-4* mutations were detected by the cosmid K04F10.

All mutant classes of *bli-4* are rescued by K04F10

To confirm that K04F10 contained all the information required for *bli-4* expression, we used a transgenic nematode strain that carries this cosmid to test for rescue of the various mutant phenotypes. The transgenic strain (kindly provided by J. McDowall, University of British Columbia, Vancouver) carries K04F10 as an extrachromosomal array along with the dominant selectable marker *rol-6* that causes worms to roll along their longitudinal axis (Mello et al. 1991). The K04F10-containing array (*hEx4*) was essentially used as a free duplication to provide wild-type function in a mutant background. Specifically, *hEx4* was transferred by genetic crosses to lethal strains carrying a *bli-4* mutation and flanking markers. Successful rescue of the lethal phenotype was indicated by the appearance of viable morphological mutants in the resulting outcrossed progeny. Normally the flanking markers would be masked by the lethal phenotype. Using this approach, transfer of the extrachromosomal array to the noncomplementing class II lethal mutant *h1010* [genotype *unc-63 bli-4(h1010) unc-13; szT1*] resulted in the generation of Unc-63 Unc-13 Rol-6 progeny. In addition, transfer of *hEx4* to the complementing class III lethal *s90* [genotype *bli-4(s90) unc-13; sDp2*], gave rise to Unc-13 Rol-6 progeny. These results showed that the cosmid is able to rescue the lethal phenotype of both classes of *bli-4* mutation.

Transfer of *hEx4* to blistered *e937* animals resulted in the establishment of several roller lines. The progeny from each of these lines consisted of blistered non-Rol-6 and nonblistered Rol-6 animals. Because extrachromosomal arrays segregate in a non-Mendelian fashion (Mello et al. 1991), we would expect to see blistered non-Rol-6 progeny in the roller lines because they are homozygous for the *bli-4(e937)* mutation. We have maintained these strains for many generations and have never observed blistering of the Rol-6 animals, indicating that the cosmid array also rescued the blistered phenotype of *e937*. Transfer of an extrachromosomal array containing only the *rol-6* plasmid to *e937* animals failed to rescue the blistered phenotype. These experiments showed that K04F10 can rescue the mutant phenotype for all classes of *bli-4* mutation. Therefore, we concluded that the cosmid contains the *bli-4* gene.

Isolation and characterization of *bli-4* cDNAs

The 1.3-kb *EcoRI* genomic DNA fragment from K04F10 that identified the DNA rearrangement in *h1010* was

Figure 1. Characteristic phenotypes of the three *bli-4* mutant classes using Nomarski photomicrographs. (A) Wild-type adult. (B) *e937* homozygous (class I) adult hermaphrodite showing the blistered cuticle. (C) Wild-type threefold embryos showing the typical elongated morphology. (D) An arrested *bli-4(q508)* embryo representative of the terminal phenotype associated with the class II lethal mutants. Note the vacuolated cells within the head region (arrows). (E) An *unc-13(e51)* L1 larva. (F) The single class III allele *s90* [genotype *bli-4(s90) unc-13(e51)*] showing the arrested "dumpy" phenotype depicted by ~30% of animals homozygous for the mutation. (A,B) Bar, 100 μ m; (C-F) bar, 10 μ m.

used as a probe to screen the cDNA library of Barstead and Waterston (1989) identifying six cDNA clones. Additional cDNAs were identified as part of the ongoing genome sequencing project (Waterston et al. 1992; Y. Kohara, pers. comm.). The clones were placed into four groups according to their structural similarities and have been designated blisterase A, blisterase B, blisterase C, and blisterase D (Figs. 3 and 4). Blisterase A cDNAs (six clones) encode a putative protein of 670 amino acids. A single blisterase B cDNA contains a 2419-bp insert, including a large open reading frame, which begins with the same potential ATG start codon as blisterase A, that

can encode a protein of 730 amino acids. A single blisterase C cDNA contains a 2659-bp insert, which contains an open reading frame of 2481 bp that begins with the same initiator methionine as blisterases A and B, predicting a protein of 827 amino acids. Three blisterase D clones were identified, although all represent partial cDNAs that are missing sequences at the 5' end. The longest clone begins at nucleotide 741 of blisterase B (Fig. 4A). The reverse transcriptase-polymerase chain reaction (RT-PCR) analysis indicated that the *bli-4* transcripts are *trans*-spliced to the SL1 splice leader (Krause and Hirsh 1987). The RT-PCR results, together with

Figure 2. Genetic and physical maps of the *bli-4* region. *bli-4* was mapped genetically between the N2/BO strain RFLP *hP5* and the left breakpoint of the deletion *hDf8* (see Materials and methods). This interval is spanned by 200 kb of contiguous cosmid clones, except for a small gap between K06E6 and C44D11, which is spanned by yeast artificial chromosome (YAC) clones (Coulson et al. 1986, 1988). The positions of *hP5* and the left breakpoint of *hDf8* in the physical map are indicated by shaded arrowheads. All of the cosmids shown were used to probe genomic DNA isolated from several mutants.

Figure 3. Genomic organization of the *bli-4* gene and the results of phenotypic rescue experiments. (A) A partial restriction map of ~15 kb of genomic DNA containing the *bli-4* gene is shown at the top. The structure is based on complete genomic sequencing of exons 1–12, 2 kb of genomic sequence around exon 13, and 4.2 kb including exons 14–21. The amino acid substitution in *h199*, the site of the *Tc1* insertion in *h1010*, the 366-bp deletion in *q508*, and the 3.5-kb deletion of *e937* are indicated. (Bottom) The four groups of cDNA clones. Solid boxes indicate exons; open boxes represent untranslated sequences, including the SL1 *trans*-splice leader sequence (Krause and Hirsh 1987). (B) Representation of plasmid constructs used for rescue of the blistered and lethal (both class II and class III alleles) mutant phenotypes by germ-line transformation. (+) Complete rescue; (+/-) incomplete rescue; (–) no rescue; (nd) not done. Restriction enzymes: (Xb) *Xba*I; (Xh) *Xho*I; (P) *Pst*I; (Sal) *Sal*I; (E) *Eco*RI; (K) *Kpn*I; (B) *Bam*HI. Some restriction sites used for cloning have been omitted for clarity.

data obtained by Northern blot analysis (see below), suggest that all blisterases share the same 5' sequences.

All cDNA clones are complete at their 3' ends, terminating with a poly(A) tract. However, all four groups have different 3' ends. The point of divergence is after nucleotide 2151 of the blisterase B cDNA sequence as shown in Figure 4. We determined the genomic intron/exon organization of the *bli-4* gene by partial restriction mapping and sequence analysis and found that the four cDNA groups diverge from each other at an intron/exon boundary (see Fig. 3A). This implies that the different predicted isoforms are produced by alternative splicing. An interesting characteristic common to both the blisterase B unique 3' exon and exon 18, which is specific to blisterase C, is the extremely short 3'-untranslated region (3' UTR). The 3' UTRs for these cDNAs are only 23 and 31 nucleotides long, respectively (Fig. 4), which is unusual, at least when compared with other *C. elegans* genes.

Rescue of *bli-4* mutant phenotypes by a subset of gene products

The functional activity of each BLI-4 isoform was exam-

ined by using transgenics carrying various subclones of K04F10. A number of constructs were generated that contain genomic sequences encoding one or more of the *bli-4* gene products (see Fig. 3B). Complete rescue of the class II (*h199*, *q508*) and class III (*s90*) lethal phenotypes and the blistered phenotype was obtained using the constructs that contain sequences encoding only the blisterase A isoform (pCeh226, pCeh229, and pCeh230). Complete rescue was also obtained by plasmid pCeh236, which encodes isoforms B, C, and D. In contrast, expression of blisterase B alone (pCeh238) was unable to rescue the lethal phenotypes of both class II and III mutants. However, the same plasmid rescued partially the blistered phenotype suggesting functional redundancy between isoforms A and B in adult nematodes.

bli-4 encodes *kex2*/subtilisin-like serine endoproteases

A search of the GenBank, PIR, and SWISS-PROT data bases revealed significant amino acid sequence similarity between the predicted BLI-4 sequence and members of the *kex2*/subtilisin-like family of proprotein-converting enzymes. The sequence similarity is most striking

A

→ Blisterase C 5' end
 ggtttaattaccacgaatttggagcgttcgcttcgggtcgatgcgaataaacggaataaacccgttcacccagcgaatcgtcaacattttc 100
 SL1
 taccaccatcatcagtcacccaaacggatttatattattatttagagcaatcatccacatttatctcacaacgcgtgatcggtatcgatagccgcg 200
 M R I S I G R
 7
 GATAGCATGGCAATTCGGCAGTTTAAATCGAGTTCATCTCACTTATGAACATGATTCCTTTCGGATGAAGTATAGTGCTGTGGGAACCAATA 300
 J A W Q I L A V L I A V A F T J E H D S I C D E S I G A C G E P I 40
 CATACCGTATACGTTTAGCAAAAGAGATGATGAGCTTGCACGGGATAGCTGCTGATCATGACATGATGAAGAGTATCGCTTTTGGATCTC 400
 H T V I R L A K R D D E L A R R I A A D H D M H V K G D P P L D T 73
 ACTACTTCCTTTATCATCGGAACAACAGGACGCGACATAAAGAGCGATTGTTGAACGATTGGATTCACATCCAGCGCTCGAATGGTGAAGA 500
 H Y F L Y H S E T T R T R I R K R I A I V E R L D S H P A V E W V E E 107
 ACAGCGACGAAGAGAGTCAAAAGAGATTATATCTCTGGATTAATGTTTCATCATTTCAACCCCTTCGGCGTTCGGTTTGAACCGTGATGGT 600
 Q R P K K R V K R D Y I L L D N D V H H S N P F R R S V L N R D G 140
 ACTCGTAGAGCTCAACGACGACGACGCTCCAGCAAGAAATTCATCACTTTCATGACCTTTTATAAGACGAGTGGTATTCATGGTG 700
 T R R A Q R Q Q P Q S P G E I P S L P F P D P L Y K D Q W Y L H G 174
 → Blisterase D 5' end
 GAGCAGTTGGTGGATGATGATGATGTTCTCGAAGCGGCTTCAAGGATATGAGCGAGAAATGTTTCAGTTTCGATTTCGACGATGAATCAAG 800
 G A V G G Y D M N V R Q A W L Q G Y A G R N V S V S I L D D G I D R 207
 AGATCTCTGATTGGCAAGCAATCGATCCACTCGGTCACAGCATATCAATGATCATGATGATCCACCAACCAACCAATGGAGATCAAC 900
 D H P D L A N Y D D L D D L P T O N N G D N K 240
 CATGGTCAAGATGCTGGAGAAGTAGCTGCACTCGCGGAACAACATCAATGGTGGTTCGCTTCAAGCAAAATAGGAGGATTCGTATG 1000
 N G T R C A G E V A A L A G N N Q C G V G V A F K A I I G G V R M 273
 TTGATGGAGTGTTCGGATTCTGCGAAGCTGGCTGATGTTCTTGAATCAGATCATATGATATATCTCAGCATCATGGGACCTGAAGACGCG 1100
 L D G A V S D S V E A A S L S L N Q D H I D I Y S A S W G P E D D G 307
 AAAGACTTTTATGATGGTCCAGCTTCACCGCAAGCTTTTATCGGGAATCAAGATGGCAGAGTGGAAGGAACATTTTGTATGGCCAGT 1200
 K T F D G P G L A R E A F Y R G I K N G R G K G N I F V W A S 340
 GGAACGGTGGATCAACCGACACTGATGTCAGCTGATGTTACACAACTCAGCTACACCGTTTCCATATCTTCGGCTACTTATGATAATCACAC 1300
 G N G G S S Q D S C S A D G Y T T S V Y T L S I S S A T Y D N H R 373
 CATGGTATTGGAAGATGCCATCATCAATTCACCAACATACAGTTCGCGATTTCGGTCAACGCAATGTGACCGTTGATGTTCTCGGAGGATG 1400
 P W Y L E E C P S S I A T Y T S S A D F R Q P A I V T V D V P G G C 407
 TACTGATAGCATACAGCAACTCTCGGCTGCTGCTTGGCAGCTGGAATTAATGCTGCTTGAAGGCTAATTCCTGAATTAACATGGCGGATATG 1500
 T D K H T G T S A S A P L A A G I A L A L E A N P E L T U R D M 440
 CAACATCTTGTCTTCGAACCGCACTGGAAACCACTTGAGAATATCTGGATGGTCAAGAAATGGTGTGGAGTATGGTATGAGCAATTAATTCGGT 1600
 Q N L V L R T A N W K P L E N N P G W S R N G V G R M V S N K F G 473
 ATGGTCTAATCGTGGTGGCAGTTCGTAATAGGCAACATGGAGACAGTCCGACATGATGAATCAAGCTTGTCAATTC 1700
 Y G G T G G A L V N M A K T V P E Q H I C T Y E Y R L A N P 507
 AATCTCCGCGCAATTTGGGTGCTTCCCACTTAATTTCACTTATGATGATGATGGAGTGGCAGTCCGCAACCCCTGTTTATATTTGGAACAGCTTCAA 1800
 N P R P I V G R F O L N F T L D V N G C E S G T P V L Y L E H V Q 540
 GTGCATGCTACTTTCATATCTGAAACGTCGCGATCTGAAGCTTACGCTCTTCTCACCATCGGACCCGATCGGTTCTTCTCCACACGACCAAG 1900
 V H A T V R Y L K R G D L K L T L F S P S G T R S V L L P P R P Q 573
 ATTCAATGCTAACGGATCCCAAGTGGCTTTCTTTCAGTCCCAATGGGGAAGATCCAGTGGAACTGGCTTCTCATGGTGAATCAGTCAC 2000
 D F N A N G F H K M P F L S V Q Q W G E D P R G T W L L M V E S V T 607
 CACTAATCCAGCTGCTACTTCTAGCTTCCAGATTGGACTTCTTCTTCTTATGGAATGCTGATCCAGCTCAATCAGGTGATCTGCTACTCGGCTACT 2100
 T N P A A T E T F H D W T L L L Y G T A D P A Q S G D P V Y S A T 640
 CCTGCAACATCCCAAGGATTCCTCAGCGGTTCATCACTCACTTCTCAG 2151
 P A T S Q G V L S R V H O L T S Q 657
 Blisterase A unique 3' end
 ATACTAATCAGTCCGATATCCAGCTGCTGCAACGGATGAATatttttctgttctcagctgcacagcttcatagagcttaaaattagattttattgtcc 2251
 I L I T I A I N L V V N A * 670
 ctttattcttggtttagatgtgtgtgaaactgtctccccattttctgtgctatttctcttttgacctcactatgataaactttttcagagaaaa 2351
 aaaaaaaaaaaaaa 2366
 Blisterase B unique 3' end
 GGTGATGAATTTGTGAAGAACTGCAATTCAGGAGTACATAGAGAGCTTCACTTGAATTTGGAGGATGCTGCTGAACATTAATCAATTCAT 2251
 G D E V V E R I R N H W E V T L E E S S H W N W E H A R E N K S L 690
 AAGATTGAACCTTCTCTCGTACCCATAGTTTTTATCTCTTCAAGAAATTCACCGATTTTCTGATATTTGCTGTGATTTTTCATGCGAT 2351
 Q E L N S S S R T H S F L Y S F T K F Q P I F L I L V C I F D A I 724
 TCATGCCCAATTCGCGTTTGAactataatgacttcaatttgggttaaaaaaataaaaaaataaaaaa 2424
 H R Q F A V * 730
 Blisterase C unique 3' end
 GTTGAAGAGTCTGCTCCGATCTCATTTCCAGATTTGACGTGGCTGGAATTTGTCATGATGATGAATGAATGAAGTTCACAGAAATGAGCTCAGCAACAT 2251
 V E E S A P I S F P D L T S A G N C H D E C N G G C T E S S A T 690
 CATGTTTCGCTTCAACACTTAACTCAAACTCTCGAAATAAGGAGGAGTGGATTCAAGTGTGTCAAAATGCGATGATCTACTACTTGGATGG 2351
 S C F A C K H L T Q T L R N K G G S G F K C V Q K C D D T Y Y L D G 724
 TGCAAGTGTAAAATGTGCTATCCCATTTGTCATCTTGTACGAAGCTGAGTGTGTGAACGCTGCTCGGAAGCTTTTGTCTGATGATGATGAAT 2451
 D K C K M C S S H C H T C T K A E V C E T C P G S L L L I D V D N 757
 ATGCCACACTGATCATGGAAGTGTGTGAGTCACTGCTCCAGGATGGTGTGCTGATTCAGCTGAAATTTGATTTTTCGGCGCAAAATACGAAAT 2551
 M P H Y D H G K C V E S C P P G L V A D Y E S N L V Q A K C I W R 790
 CTGGTGGACACGACAGTGTGTTGTAATTCAAAAGCCGAGCCCTTAAAGATTAAGTGAATTTGAACCTTGTGATTTAGACTTTTATTTTTC 2651
 S G R D T T V F V K F K K P S A F K D Y C N L K L V D L D F F I F S 824
 TCTCTATTTTATGTTTTTTTTTgctataaattttttttatcgaataaaaaa 2704
 L L F * 827

Blisterase D unique 3' end

GTTGAAGAGTCTGCTCCGATCTCATTTCCAGATTTGACGTGGTGAATTTGATGATGAATGAATGAGTTCACAGAACTGAGCTCAGCAACAT 2251
 V E E S A P I S F P D L T S A G N C H D E C N G G C T E S S A T 690
 CATGTTTCGCTTCAACACTTAACTCAAACTCTCGAAATAAGGAGGAGTGGATTCAAGTGTGTTCAAAATGCGATGATCTACTACTTGGATGG 2351
 S C F A C K H L T Q T L R N K G G S G F K C V Q K C D D T Y Y L D G 724
 TGCAAGTGTAAAATGTGCTATCCCATTTGTCATCTTGTACGAAGCTGAGTGTGTGAACGCTGCTCGGAAGCTTTTGTCTGATGATGATGAAT 2451
 D K C K M C S S H C H T C T K A E V C E T C P G S L L L I D V D N 757
 ATGCCACACTGATCATGGAAGTGTGTGAGTCACTGCTCCAGGATGGTGTGCTGATTCAGTGAATCTTGTCAAGTAATATCTATGGAGAA 2551
 M P H Y D H G K C V E S C P P G L V A D Y E S N L V Q A K C I W R 790
 AAGATCTTTCGCTGAGGATTAACATCAAGCGTGTGGAATGTGATCTTTCGACCTCTCATGGAAGCTTCGACCGCTCTGGTCCAATGAGCTG 2651
 K D L C G D G Y Y I N A V G K C D L C D S S C E T C T A P G P M S C 824
 TGAGAAATGTTCAAAAGGTTATGGTAAAGGATCGATTGGATCTAGACCGTGTGCTCGAAGGATCAACAAAGAGTGGCAATGTGGAGCTGCTCC 2751
 E K C S K G Y G K G S I G Y C R P C C P E G S T K S W Q C E D C S 857
 AAGCGGATCTACACTTTTGTGATTTCAATAATCATCTGGATTGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG 2851
 K P D P T L L I D S N K S S G F G L M F W I V V S L I A A C G I C 890
 CCGTAAAGAGTGTCAAGTGAGCAAGAGCTCAACGATGATATGCGCGCTGCTCAATATAATGCCAAAGTGGTGTATCAATTTAGGAGGACA 2951
 A C K K C A S E T K S S N V E Y A P L A Q Y N A T I N G A I N L G A H 924
 CACTGACGATGAAGAGGATGATGAGGATGAAGTATTTGTAACCTCAATTTGTTAAaccaccccttcaaaattcattgttttaattgtattttct 3051
 T D D E D D D E D E V F V N E Y I V * 942
 gccactcttttctgtatgctgttgaactcgggttaattgtttctttttcaaaattttaaaccgtgaacgttttcaagtgcccttttcatgtgtattg 3151
 tattgtttcttttctttatcagggtttatttttatataccgtactgtttccattatgaatagattgttgaatttaaaaaa 3237

B

Blisterase C variant coding sequence

nt 2506 GGT GAT Tgt aag ttt tat aag agt ttc aga ata aaa agt taa tctccag AC GCT GAA nt 2520
 A D C K F Y T K S F R I K S * Y A E
 exon 17 intron 17 exon 18

Figure 4. Nucleotide and predicted amino acid sequence of the *bli-4* cDNAs. (A). The common and unique 3' sequences of each *bli-4* gene product is presented. The 5' ends of representative blisterase A, C, and D cDNAs are indicated by right-angled arrows. SL1 is the splice-leader sequence (Krause and Hirsh 1987). Results obtained using Northern blot analysis suggest that blisterase D has the same 5' end as blisterase B. The putative amino-terminal signal sequence is underlined. The *kex2*/subtilisin-like protease domain is double underlined, as is the hydrophobic amino acid sequence in the blisterase D specific 3' end. The potential cell attachment site Arg-Gly-Asp (RGD) is overlined and underlined. The potential autocatalytic cleavage sites (Arg-X-Lys/Arg-Arg) have been boxed. Upward open arrows denote catalytically important amino acid residues Asp, His, Asn, and Ser. Solid downward arrowheads denote the position of intron/exon boundaries. Polyadenylation sites are underlined. The A → T transversion identified as the lesion in *h199* causing the His-127 → Leu substitution is indicated. The site of the *Tc1* insertion identified associated with the *h1010* mutation is labeled by a downward arrow (between nucleotides 1696 and 1697). Thirty-seven coding nucleotides that are deleted in the class II mutant *q508* are indicated within the square brackets. (B) The putative carboxyl terminus of the variant blisterase C transcript. Amino acids potentially encoded within intron 17 are shown. The splice donor and acceptor are underlined.

within the kex2/subtilisin-like protease domain (Figs. 5 and 6). hFURIN shows the most extensive conservation with the blisterases in this region, exhibiting 72% sequence identity (Figs. 5 and 6). The blisterase protease domain includes catalytically important amino acids Asp-202, His-241, Asn-342, and Ser-415, which are present in all members of the kex2/subtilisin-like endoprotease gene family (with the exception of PC2).

Although the sequence similarity is restricted primarily to the protease domain, the blisterase proteins share structural features outside this region with the other kex2/subtilisin-like endoproteases (Fig. 6). These conserved structural similarities include pairs of basic residues on the amino side of the protease domain that are potential sites of activation by autoproteolytic cleavage. This has been demonstrated in the bacterial subtilisins

(Power et al. 1986; Ikemura and Inouye 1988) and, recently, for hFURIN (Hosaka et al. 1991; Molloy et al. 1992, 1994). The conservation of a tripeptide Arg-Gly-Asp (RGD) motif representing the minimal recognition sequence required for cell attachment located in a similar position relative to the active site [with the exception of Kex2p, which has the sequence Arg-Gly-Thr at this position (Fuller et al. 1988)] is also conserved. Furthermore, certain members of the kex2/subtilisin-like family, including hFURIN (Roebroek et al. 1986), PACE4 (Kiefer et al. 1991), PC5/PC6 (Lusson et al. 1993; Nakagawa et al. 1993), Dfurin-1CRR (Roebroek et al. 1994), and Dfurin-2 (Roebroek et al. 1992), have a Cys-rich region toward the carboxyl terminus [Kex2p is Ser-rich in this region (Fuller et al. 1988)]. Blisterases C and D both encode a Cys-rich region. Blisterase C contains a

Figure 5. Alignment of the protease domain of BLI-4 (residues 162–443), with PACE4 (residues 161–448; Kiefer et al. 1991), hFURIN (human furin; residues 114–396; Wise et al. 1990), hPC2 (human PC2; residues 122–412; Seidah et al. 1990), mPC3 (mouse PC3; residues 122–410; Seidah et al. 1991), Dfurin1 (*Drosophila* furin; residues 333–615; Roebroek et al. 1994), and Kex2p (yeast; residues 134–411; Mizuno et al. 1988). Residues that are identical to the BLI-4 sequence have been boxed and gaps introduced to give maximum sequence alignment. Shaded residues indicate the catalytically important amino acids Asp, His, Asn, and Ser.

Figure 6. Schematic structural comparison between the BLI-4 products and other members of the kex2/subtilisin-like family of endoproteases. Structural features and relative lengths of the predicted BLI-4 isoforms, mPC4, mPC3, hPC2, hFURIN, PACE4, hPC5/hPC6, Dfurin-1CRR, Dfurin-2, and Kex2p polypeptides are shown. Secretion signal peptides are shown as shaded; protease domains are shown stippled with the positions of the catalytically important amino acids Asp, His, and Ser indicated by D, H, and S; the Cys-rich regions are depicted as hatched boxes; transmembrane domains are shown as solid boxes. Ovals indicate potential N-linked glycosylation sites. The potential autocatalytic cleavage sites are shown as vertical bars on the amino side of the protease domain. The numbers within the protease domains represent the percentage of identical residues when compared with the BLI-4 isoforms.

single Cys-rich region (encoded by exon 17), whereas blisterase D has two Cys-rich domains encoded by exons 17 and 19.

Hydrophobicity analysis [Kyte and Doolittle 1982] of the blisterases revealed two major hydrophobic domains. The first is a short hydrophobic domain at the amino terminus with features typical of a signal sequence. A second hydrophobic sequence is located near the carboxyl terminus of blisterase D and is followed by a highly acidic 48-amino-acid domain [Figs. 4 and 6]. Similar hydrophobic domains followed by acidic residues are found in Kex2p, hFURIN, Dfurin-1-CRR, and Dfurin-2. Taken together, this information suggests that the hydrophobic domain of blisterase D is likely to be a transmembrane domain. The remaining BLI-4 isoforms all contain several hydrophobic residues located toward the carboxyl termini, although the hallmarks typical of a transmembrane domain are not apparent.

The overall sequence similarity of the *bli-4* gene products with members of the kex2/subtilisin-like family of proprotein convertases implies a conserved functional activity in the nematode. Alternative splicing of *bli-4* transcripts results in the putative production of enzymes with structurally different carboxyl termini, specifically a transmembrane domain-containing gene product and at least three proteins lacking a transmembrane domain.

Expression pattern of a *bli-4/lacZ* fusion gene

A *bli-4/lacZ* promoter fusion gene was constructed to analyze the expression pattern of *bli-4*. An ~5-kb *Xba*I-*Cl*aI genomic fragment (see Materials and methods) containing sequences at the 5' end of the *bli-4* gene was inserted into the *lacZ* expression vector pPD21.28 [Fire et al. 1990]. Transgenic animals carrying the construct were isolated and examined by staining with X-gal. Examination of adult and larval transgenic animals re-

vealed *lacZ* expression in all hypodermal cells, the vulva, and the ventral nerve cords [Fig. 7A,B]. Expression was first observed in embryos at the twofold stage [Fig. 7C] just before the developmental time of arrest seen for the *bli-4* lethal mutants. Staining appears to be localized to the nuclei of the hypodermal cells in the embryo.

Deletion of sequences in *e937* eliminates blisterase A expression

Restriction fragment length polymorphism (RFLP) analysis revealed a 3.5-kb deletion within the *bli-4* gene in animals homozygous for the *e937* mutation. To characterize the mutation further, the deletion endpoints were sequenced and demonstrated to remove the last exon encoding the unique 3' end of blisterase A (see Fig. 3A). The deletion was expected to result in the loss of expression of this isoform but not affect expression of the other *bli-4* gene products. We examined expression of the *bli-4* gene in *e937* homozygotes by Northern blot analysis. Poly(A)⁺ RNA isolated from both wild-type N2 animals and the mutant *e937* strain was probed using a restriction fragment common to all of the blisterase cDNAs. Four RNA species were observed in the wild-type lane [Fig. 8A], corresponding to RNAs of 2.3, 2.4, 2.7, and 3.2 kb. The RNA species were identified tentatively by comparing the lengths of the transcripts with the characterized cDNAs (see Fig. 4). By this criteria we assigned identity of the 2.3-kb transcript as that for blisterase A, the 2.4-kb transcript corresponding to blisterase B, the 2.7-kb transcript as blisterase C, and the 3.2-kb message encoding blisterase D. Hybridization of the "common" probe to *e937* poly(A)⁺ RNA identified only the 2.4-kb and 3.2-kb transcripts [Fig. 8A]. This result indicated that the 2.3-kb message, whose length is consistent with that predicted for the blisterase A cDNA, is missing in *e937* animals. cDNAs isolated for blisterase C predict a

Figure 7. Expression pattern of a *bli-4/lacZ* promoter fusion gene. Expression of *lacZ* was observed in all hypodermal cells, the vulva, and the ventral nerve cords. Shown here are examples of staining in an adult hermaphrodite (A) and an L3 larva (B). *lacZ* expression is first observed in late embryogenesis at about the twofold stage and is confined to hypodermal cells (C). Arrowheads in A represent expression within the hypodermal seam cells; the arrow indicates staining of the vulva.

message of 2.7 kb for this isoform. The 2.7-kb message that hybridizes to the common probe is the correct size predicted for blisterase C, yet this transcript is missing in *e937* RNA. Because the exons specific to this isoform lie outside of the deletion breakpoints we would predict that expression of blisterase C would not be affected in *e937* animals. Therefore, we suspected that the 2.7-kb message does not correspond to blisterase C. The identity of the 2.7-kb transcript is unclear, although it may represent an alternative blisterase A message that uses a downstream polyadenylation site. However, we cannot rule out the possibility that the transcript encodes a fifth *bli-4* gene product.

The 3.2-kb RNA has the size predicted for blisterase D, assuming that the D-specific message has the same 5' end as blisterase B. This was confirmed by rehybridizing the membrane with a probe containing sequences specific for blisterases C and D. As expected, the 3.2-kb message hybridized to the probe (Fig. 8B), confirming the identity of the transcript as corresponding to blisterase D. A 2.7-kb transcript predicted to encode blisterase C was not observed using the blisterase C/D-specific probe, even after prolonged exposure (data not shown). Unfortunately, the low abundance of the 2.4-kb and 2.7-kb RNAs and the small size of the unique exons for blisterases A, B, and C for use as probes, made it difficult to establish unequivocally to which of the messages the various cDNAs correspond, at least by Northern blot analysis.

To establish further the expression patterns of blisterases A, B, and C in *e937* animals, we used the technique of RT-PCR. First-strand cDNA was synthesized from total RNA isolated from wild-type N2 and *e937*

animals. cDNA was then amplified using a sense primer corresponding to nucleotides 2088–2105 (of the blisterase B sequence shown in Fig. 4) that lie within exon 12 (a coding exon incorporated in all of the *bli-4* gene products), in combination with an antisense primer specific for each BLI-4 isoform. Primers that amplify a 220-bp product from the *C. elegans* ubiquitin-like gene (*ubl*) (Jones and Candido 1993) were also included as a positive control. The results obtained for RT-PCR are shown in Figure 8C. Products of the expected lengths for blisterase A (145 bp, lane b), blisterase B (195 bp, lane d), and blisterase C (514 and 556 bp, lane f) were obtained using wild-type RNA. The 556-bp blisterase C amplification product is the result of incomplete processing of the message and contains intron 17. The blisterase C variant encoded by this transcript can potentially encode another BLI-4 product as depicted in Figure 4. Both the expected blisterase B (lane e) and blisterase C (lane g) products were amplified using *e937* RNA. No blisterase A product was amplified from *e937* RNA (lane c), confirming that expression of this isoform is eliminated in *e937* animals.

The results from Northern blot analysis and RT-PCR confirmed that blisterase A expression is eliminated in *e937* homozygotes because of the deletion of the 3' exon specific to this isoform. Expression of blisterases B, C, and D is not eliminated by the mutation.

Characterization of molecular lesions in three class II lethal mutants

Restriction analysis of *h1010* genomic DNA identified a putative Tc1 insertion within the wild-type 1.3-kb *EcoRI*

Figure 8. Northern and RT-PCR analysis of *bli-4* expression in N2 and *e937* animals. Poly(A)⁺ RNA from wild-type N2 and *e937* animals was hybridized with a 480-bp *Clal*-*AccI* fragment that is common to all of the blisterase cDNAs (A). Transcripts that hybridized to the probe in the N2 lane (arrowheads) are 2.3, 2.4, 2.7, and 3.2 kb. Both the 2.3- and the 2.7-kb messages are missing in the *e937* lane. (B) The same membrane hybridized with a probe specific for both blisterases C and D, indicating that the 3.2-kb message corresponds to the blisterase D cDNA. To standardize RNA levels, the membrane was rehybridized with a probe containing sequences for the *S*-adenosyl homocysteine hydrolase (AHH) gene that recognizes a 1.6-kb transcript (inset under B). The numbers at left of A represent the relative position of RNA molecular size markers (GIBCO-BRL RNA ladder). (C) RT-PCR analysis of blisterases A, B, and C expression in N2 and *e937* animals. Lanes b, d, and f show amplification products obtained using N2 template RNA, and lanes c, e, and g, using *e937* template. Products obtained using primers specific for amplification of blisterase A are shown in lanes b and c, amplification of blisterase B in lanes d and e, and amplification of blisterase C in lanes f and g. The 220-bp product seen in all lanes is the result of amplification using primers designed from the *ubl* gene, included as a positive control. Lanes a and h contain a 100-bp DNA ladder (GIBCO-BRL).

fragment. We verified this interpretation by determining the exact location of the transposon insertion by PCR amplification using *bli-4*- and *Tc1*-specific primers. Sequence generated from the amplified product showed

that the *Tc1* element had inserted into exon nine, resulting in the disruption of the *bli-4* coding region (see Figs. 3A and 4). Exon nine is common to all of the *bli-4* gene products (see Fig. 3A). A second class II lethal allele, *q508*, was isolated after mutagenesis with trimethylpsoralin and UV (kindly sent to us by Lisa Kadyk and Judith Kimble), mutagens known to cause a wide range of deletions in *C. elegans* (Yandell et al. 1994). We searched *q508* genomic DNA for deletions within the first 12 exons common to all of the *bli-4* gene products. This was accomplished using a series of primers, which when used for PCR amplification, generate overlapping products that cover the entire common region. Amplification of exons 11 and 12 using *q508* template DNA yielded a product smaller than that generated from wild-type template (data not shown). Comparison of the amplified DNA sequences revealed a 366-bp deletion that results in the removal of the splice acceptor and 37 nucleotides from exon 12 (see Figs. 3A and 4). In a third class II mutant, *h199*, an A → T transversion was identified that changes the codon for His127 (CAT) into Leu (CTT). The *h199* lesion resides within exon four, which encodes a portion of the putative amino terminus, proximal to the protease domain (see Fig. 4).

All three mutations identified for the class II lethal mutants reside within coding exons common to all of the *bli-4* gene products. On the basis of these findings, we propose that these lethal mutants affect the activity or expression of all isoforms.

Discussion

We have cloned the *bli-4* locus by positioning the gene using the physical map of the *C. elegans* genome and have shown that it encodes members of the kex2/subtilisin-like family of proprotein cleavage enzymes. Our results demonstrate that *bli-4* is contained within the cosmid K04F10, based on the detection of RFLPs associated with two mutations, and the ability to rescue fully the lethal phenotype of the class II lethal alleles, *h1010*, *h199*, and *q508*, the class III lethal allele *s90*, and the blistered phenotype of *e937*. The high degree of conservation within the catalytic domain suggests a structural and functional homology with other members of the proprotein convertase family.

bli-4 encodes structurally diverse endoproteases

bli-4 is an example of a proprotein convertase gene in which two types of enzyme arise from the same gene through alternative splicing. Structurally, the kex2/subtilisin-like convertase family can be divided into two classes based on the presence or absence of a transmembrane domain near the carboxyl terminus. Blisterase D falls into the class that contains a transmembrane domain, along with Kex2p, hFURIN, Dfurin-1 (all four isoforms), and Dfurin-2. Blisterases A and B belong to the class of endoproteases that do not have a transmembrane domain, with an overall structural similarity to PC1/PC3, PC2, and PC4. Similarly, blisterase C resembles

PC5/PC6 and PACE4 most closely. Although alternative splicing in other proprotein convertase genes has been reported, only *Dfur-1* produces gene products that differ markedly in structure (Roebroek et al. 1992, 1994). At least two *Dfur-1* isoforms show a nonoverlapping, restricted expression pattern (Roebroek et al. 1994). It is possible that the different proteins encoded by both the *bli-4* locus and *Dfur-1* perform distinct physiological roles in their respective organisms that, in mammalian cells, are fulfilled by many individual convertase genes.

The e937 mutation eliminates the blisterase A isoform

We have demonstrated that the *e937* mutation is a 3.5-kb deletion within the *bli-4* gene that removes the 3' exon unique to blisterase A. Northern blot and RT-PCR analysis shows that the message encoding the blisterase A isoform is missing in *e937* animals, although a second less abundant transcript of 2.7 kb is also not observed (Fig. 8). The elimination of expression of the blisterase A isoform is consistent with what would be expected from the deletion breakpoints. Blisterase A appears to be required for the normal production or maintenance of the adult cuticle as evidenced by the viable blistered phenotype of *e937*.

Northern blot analysis using the common probe showed that blisterases B and C are normally either expressed at very low levels or in a short developmental window. Whether the low abundance of these messages is a function of the short 3' UTRs found on both isoform cDNAs remains to be determined. Detection of an unprocessed blisterase C transcript by RT-PCR suggests inefficient splicing, although intron 17 has the capacity to encode a variant C isoform. The sequence of the splice acceptor for intron 17 (TCTCCAG) is unusual when compared with most *C. elegans* genes (TTTTCAG) (Blumenthal and Thomas 1988). This may suggest a potential mechanism for controlling the expression of the blisterase C isoform, further resulting in the low abundance of this transcript.

The blistered phenotype is incompletely penetrant. The number of individuals that exhibit blisters in a given population is ~90%, and this fraction remains a heritable feature of *e937* homozygotes, regardless of their phenotype. Because *C. elegans* is hermaphroditic and strains are isogenic, incomplete penetrance would appear to correlate with the nature of the mutation in this complex gene. Although the molecular basis of penetrance is poorly understood, our preliminary analysis of the *e937* mutation may provide a first step toward determining the underlying mechanisms responsible for this phenomenon, at least with regard to *bli-4*. One possible explanation for the incomplete penetrance observed in *e937* homozygotes is that deletion of the blisterase A 3' unique exon causes the *bli-4* precursor RNA to be spliced instead to the 3' exons of blisterases B, C, and D or combinations thereof. In this case, the alternative isoforms could compensate partially for the loss of blisterase A activity in *e937* homozygotes. We hypothesize that there exists a functional redundancy between the

individual BLI-4 convertases. An observation consistent with such a hypothesis is that the blistered phenotype is fully penetrant in heteroallelic combinations of *e937* with most noncomplementing class II lethal alleles. If expression of the *bli-4* gene products is eliminated completely by a class II mutation, there would be less blisterase B, C, and/or D activity to compensate for the loss of blisterase A. The reduction in the compensating isoforms would result in animals that are more severely blistered, consistent with our observations. Rescue of both the lethal and blistered mutant phenotypes by constructs that encode only blisterase A and plasmid pCeh236 (which can encode blisterases B, C, and D) also suggests functional redundancy between the various BLI-4 isoforms. Rescue of the lethal mutants by constructs that only encode blisterase A is contradictory to what is observed for the *e937* mutation. The genetic data indicate that loss of blisterase A expression is not essential for development. One explanation for rescue by these plasmids is that expression from the extrachromosomal array may be aberrant.

The phenotype associated with the *e937* mutation suggests that blisterase A convertase activity is required for the production or maintenance of the adult cuticle. It could be that the cuticular collagens are substrates for cleavage by this isoform. Although no known mutations within *C. elegans* collagen genes result in a blistered phenotype, many of the cuticle collagens (e.g., *rol-6* and *sqt-1*) contain an Arg-X-Lys/Arg-Arg motif within the amino termini (Kramer et al. 1990; Kramer and Johnson 1993). Recently, it has been shown that mutations within these motifs disrupt normal collagen function implying that a kex2/subtilisin-like convertase activity may be required for processing of these cuticle collagens (Yang and Kramer 1994). Other candidate substrates for processing by the blisterases include five other genes in which mutations result in blistering of the cuticle (Brenner 1974; Park and Horvitz 1986). As yet, the molecular nature of these five blister mutants remains unknown.

Class II lethal mutants contain mutations in the common coding region

Molecular characterization of the lesions responsible for three class II lethal alleles has revealed that the mutations reside within the first 12 exons that are common to all of the BLI-4 isoforms. The *h1010::Tc1* insertion site is within exon nine, suggesting that the presence of the transposon disrupts expression of all of the blisterases. The mutation *q508* is a deletion that affects exon 12. We would predict that this lesion results in the complete lack of blisterase activity owing to the truncation of the *bli-4* gene products. The deletion removes the splice acceptor and coding sequences from exon 12. Splicing of the upstream region to the unique 3' exons, if it occurs, would effectively cause all of the remaining coding sequences to be out of frame. Thus, *q508* is a good candidate for a null mutation. The *h1010* and *q508* mutations both reside within a region of the blisterases that is uniquely conserved among all of the identified members

of the kex2/subtilisin-like family and has been designated the "middle" domain (Van de Ven et al. 1990), or the "P-domain" in Kex2p (Fuller et al. 1991). Deletion analysis of Kex2p mutants has shown that the P-domain is essential for processing activity and may be required for either proper folding of the protease domain or recognition of substrates (Gluschankof and Fuller 1994). *h199* contains an amino acid substitution that would also affect all *bli-4* gene products. At present, we are unclear as to the exact mechanism by which this lesion reduces blisterase activity. A systematic search for mutations within the unique 3' exons of the class III mutant, *s90*, has failed so far to reveal any lesions. Because the *s90* mutant phenotype was rescued by K04F10 and subclones of the cosmid, the responsible mutation may be elsewhere within the *bli-4* locus.

Preliminary analysis of animals homozygous for the lethal alleles of *bli-4* suggests that development does not arrest until the end of embryogenesis. This could indicate that *bli-4* is not required until later stages of embryonic development, which is consistent with the earliest time of expression observed for the *bli-4/lacZ* fusion gene. It is also possible that a maternal endowment of *bli-4* activity provides mutants with sufficient blisterase function to survive to this stage. The similarity of the *bli-4* lethal mutant phenotype with that shown by cuticle-defective mutants suggests that blisterase activity is required for elongation of the nematode embryo.

Of the other members of the kex2/subtilisin-like gene family, only *KEX2* was identified by genetic analysis; null mutations in *KEX2* result in the inability to process the α -mating factor pheromone and the K1 killer toxin (Leibowitz and Wickner 1976; Wickner and Leibowitz 1976). *KEX2* is not required for viability in yeast, indicating that Kex2p does not play an essential intracellular role. In contrast, potential null mutations in *bli-4* result in developmental arrest near the end of embryogenesis before hatching. Such evidence demonstrates that *bli-4* is essential to development, and implies the existence of blisterase substrates required for embryogenesis. Such substrates could include both structural molecules and peptide signals. Although essential function involving a secreted protein derived from a proprotein has not yet been described in *C. elegans*, the disruption of development by *bli-4* mutants implies the existence of such interactions.

Materials and methods

Nematode strains and culture conditions

Worms were maintained on NGM growth media as described by Brenner (1974). Nomenclature used conforms to the uniform system for *C. elegans* (Horvitz et al. 1979). Nomenclature for translocations conforms to that used by McKim et al. (1988). The *bli-4(e937)* allele was isolated using ³²P (Babu 1974; Brenner 1974). Most of the class II lethal mutants were identified previously from a set of lethal mutations induced in ethylmethane sulfonate (EMS) screens (Howell et al. 1987; Peters et al. 1991; C. Thacker, M. Srayko, and A.M. Rose, unpubl.). Additional alleles of this class not discussed in this study include *h42*

(Howell et al. 1987), *h254*, *h384*, *h427*, *h520*, *h699*, *h791* (Peters et al. 1991), *h670*, and *h1403* (C. Thacker, M. Srayko, and A.M. Rose, unpubl.). *h1010* was a spontaneous allele isolated in a precomplementation screen for *bli-4* alleles in a *mut-6* background (Peters et al. 1991). *h1010* was balanced by the translocation *szTI(I;X)*, which completely suppresses recombination on the left-half of LG I (Fodor and Deak 1985; McKim et al. 1988) in a strain of the genotype *szTI(I;X) [lon-2]/unc-63(e384) bli-4(h1010) unc-13(e450) I*. The lethal alleles of *bli-4* were balanced by *sDp2(I;f)*, which is a free duplication of the left-third of LG I (Rose et al. 1984). These *bli-4* lethal alleles were maintained in strains having the genotype *sDp2; dpy-5(e61) bli-4(hx) unc-13(e450) I*. The class III allele *s90* was isolated by Rose and Baillie (1980) and was maintained as *sDp2; bli-4(s90) unc-13(e51) I*. JK1723, genotype *bli-4(q508)/dpy-5(e61) unc-87(e843) I*, was isolated by L. Kadyk and J. Kimble (University of Wisconsin, Madison) after mutagenesis with trimethylpsoralen and UV. Other strains used in this study were KR2997, genotype *sDp2; dpy-5(e61) bli-4(h199) unc-13(e450)*; KR2728, genotype *sDp2; dpy-5(e61) bli-4(s90) unc-13(e450)*; KR2572, genotype *sDp2; dpy-5(e61) bli-4(q508) I*; KR2276, genotype *unc-11(e47) I; hEx4*, and KR2872, genotype *bli-4(e937) I; hEx6*.

Aligning the physical and genetic maps for the bli-4 region

bli-4 was mapped with respect to *dpy-5* and *unc-13* by RFLP analysis as described by Baillie et al. (1985). *bli-4* was placed ~0.3 map units to the right of the polymorphism *hP5*. The cosmid corresponding to the *hP5* site is T09H3. The DNA density in this region was ~500 kb per map unit (Starr et al. 1989), predicting that *bli-4* would be located ~150 kb to the right of *hP5* (see Fig. 2). The deficiency *hDf8* maps to the right of *bli-4*. The left breakpoint of *hDf8* was mapped to the cosmid C44D11 by restriction analysis. Together, these results positioned the *bli-4* coding region to cosmids located to the right of T09H3 and to the left of C44D11 on the physical map (Fig. 2). To determine the exact position of *bli-4* sequences, cosmids that span this region together were used to probe for RFLP in genomic DNA prepared from various *bli-4* mutants.

Rescue of the blistered and lethal phenotypes

We used a transgenic nematode strain, KR2276 (kindly provided by J. McDowall), that carries an extrachromosomal array designated *hEx4*, composed of two overlapping cosmids K04F10 and C29F10 and the plasmid pRF4. The cosmid C29F10 overlaps K04F10 by 95% (data not shown) and was included to facilitate homologous recombination between the various clones to generate the array. The plasmid pRF4 contains the *rol-6* gene bearing the semidominant mutation *su1006* that confers a right-handed Roller phenotype to animals that carry the array (Mello et al. 1991). As a control, all the genetic crosses outlined below were repeated using a transgenic strain (KR2872) that carries only the plasmid pRF4 as an extrachromosomal array (*hEx6*).

Rescue of e937 Homozygous *e937* males were crossed with KR2276 Rol-6 hermaphrodites. All Rol-6 hermaphrodites obtained in the F₁ were isolated and allowed to self-fertilize. Non-Unc-11 Rol-6 hermaphrodites were isolated until no Unc-11 animals were recovered indicating homozygosity for *e937*. Several Rol-6 lines were established that segregated Rol-6 (wild-type) and Bli-4 progeny. No Bli-4 Rol-6 animals have ever been observed over many generations. However, Bli-4 Rol-6 animals were obtained using the control transgenic strain in parallel experiments.

Rescue of h1010 Spontaneous Lon-2 males of the genotype *szT1(I;X) [lon-2] unc-63 bli-4(h1010) unc-13; I* were crossed to KR2276 Rol-6 hermaphrodites. Successful rescue was determined by the presence of Unc-13 hermaphrodites in the F₂ generation. *unc-13* is epistatic to *unc-63* and *rol-6*. To verify the presence of the *rol-6* marker, rescued animals were mated with wild-type males. Rol-6 animals were obtained in the F₁ progeny. No Unc-13 progeny were obtained using the control Rol-6 array. The presence of the *h1010::Tc1* within the Unc-13 animals was verified by PCR using primers KRp36 and p618 (see below).

Rescue of q508 and s90 Heterozygous males of the genotype *dpy-5 bli-4(q508)/+ +*, or *bli-4(s90) unc-13/+ +* were crossed with KR2276 Rol-6 hermaphrodites. All Rol-6 hermaphrodites in the F₁ generation were isolated and allowed to self-fertilize. Successful rescue was determined by the presence of Dpy-5 progeny in the case of *q508* or the presence of Unc-13 progeny in the F₂ generation for *s90*. No such progeny were obtained using the control transgenic strain. The presence of the *q508* deletion within the rescued Dpy-5 animals was verified by PCR using the primers KRp46 and KRp47 (see below). A similar strategy was used for rescue experiments using the K04F10 subclones shown in Figure 3B. Heterozygous males of the genotype *dpy-5 bli-4(q508)/+ +*, *dpy-5 bli-4(s90) unc-13/+ +* or *dpy-5 bli-4(h199) unc-13/+ +* were crossed to Rol-6 hermaphrodites bearing pRF4 and the plasmid constructs. Successful rescue was determined by the presence of Dpy-5 progeny in the case of *q508* or the presence of Dpy-5 Unc-13 progeny in the F₂ generation for *s90* and *h199*. Individual lines for each rescue were established and observed to give a fraction of dead progeny, resembling the *bli-4* lethal phenotypes. Outcrossing of the putative rescued lines produced Rol progeny. Partial rescue of blistering by pCeh238 was evidenced from four independent stable transgenic lines of genotype *bli-4(e937); hEx46* to 49 (pCeh238 + pRF4). The percentage of Rol-6 animals that blister in each of these lines is 43% (79/185), 8% (41/523), 17% (17/101), and 3% (7/206), respectively, indicating incomplete rescue of the blistered phenotype.

Isolation and characterization of *bli-4* cDNA clones

cDNA clones were isolated from a λ ZAP library (Barstead and Waterston 1989) or provided by Yuji Kohara (National Institute of Genetics, Mishima, Japan; cDNA clone groups YK849 and YK949). The cDNA sequence was obtained from nested exonuclease III deletions as described by Henikoff (1984) and various fragments subcloned into pBluescript (Stratagene). Sequencing was performed using either Sequenase (U.S. Biochemical) or the Amplitaq DNA polymerase sequencing kit (ABI) and a Perkin-Elmer Cetus thermal cycler. Some sequence was obtained using an ABI model 373A automated sequencing machine. DNA and protein data base searches were performed using the FASTA program (Pearson and Lipman 1988). The PCgene (Intelligenetics) package of programs was used to identify the secretion signal peptide, hydrophobic domain, and potential glycosylation and cell attachment sites.

K04F10 subclones

Subclones used for transgenic rescue of *bli-4* phenotypes presented in Figure 3B are as follows. pCeh226 contains a 13.5-kb *XbaI*-*XhoI* insert containing exons 1–13. pCeh226 was digested with *SalI* and recircularized to obtain pCeh229. pCeh230 contains the same sequences as pCeh229 except for the deletion of a 1.8-kb *PstI*-*EcoRI* fragment within intron 12. Subclones pCeh226, pCeh229, and pCeh230 encode blisterase A. pCeh238

contains a 10.3-kb *XbaI*-*EcoRI* fragment (including exons 1–12) fused to a 0.7-kb *BamHI*-*KpnI* fragment that contains exons 14 and 15. This construct encodes blisterase B. pCeh236 contains exons 1–12, and 14–21 and can encode blisterases B, C, and D. The construction of pCeh239 is discussed below. These constructs were coinjected with pRF4 (1:1 ratio) into the germ line of *e937* homozygotes (or N2 animals for pCeh239). Stable transgenic lines were recovered and used for subsequent crosses to lethal-bearing strains discussed above. Rol-6 hermaphrodites containing pCeh239 (see below) were crossed to *e937* homozygous males. *Bli-4* Rol-6 animals exhibiting approximately the same penetrance as *e937* homozygotes in the F₂ generation indicated failure of pCeh239 to rescue blistering.

Construction and expression of the *bli-4/lacZ* fusion

A 5-kb *XbaI*-*Clal* fragment containing the putative promoter region of *bli-4* was cloned into the *XbaI*-*BamHI* sites of the *lacZ* expression vector pPD21.28 (Fire et al. 1990). The *Clal* site in the *bli-4* gene occurs 10 nucleotides after the putative initiator methionine codon in exon 2. This plasmid was designated pCeh239 and used to transform N2 hermaphrodites as described above. Transgenic Rol-6 lines were fixed and stained with X-gal according to the procedure described by Fire et al. (1990).

PCR amplification of the *h1010::Tc1* and *q508* mutations

Template DNA from individual arrested embryos homozygous for each of the mutant *bli-4* alleles was extracted as described (Barstead et al. 1991). The PCR conditions used to amplify the *Tc1* insertion in *h1010* were as follows: 94°C 45 sec; 50°C 30 sec; 72°C 1 min for 30 cycles. The sequence of the primers used for the amplification were KRp36 (5'-CTATAAACCCCTTAATTG TC-3') (*bli-4* specific: anneals to a sequence within intron 7) and p618 (5'-GAACACTGTGGTGAAGTTTC-3') (*Tc1* specific). p618 was a gift from B. Williams (Washington University School of Medicine, St. Louis, MO). The PCR conditions used to amplify the deletion from DNA extracted from homozygous *q508*-arrested embryos were the same as above except that the annealing temperature was 56°C. The primers used to amplify the deletion were KRp46 (5'-ATGCTACTGGTCAGTTTTC-3') (anneals to a sequence within intron 10) and KRp47 (5'-ATTCTATCCGAATCCTCCGA-3') (anneals to a sequence within intron 12). The PCR products obtained using these conditions were cloned into the pBluescript vector and sequenced using Sequenase version 2.0.

Detection of the *h199* mutation

h199 was detected by a systematic search through exons 1–10 by heteroduplex mismatch detection using the mutation-detection enhancement (MDE) system (AT Biochemicals). Individual blistered *h199/e937* animals were used as template for independent PCR reactions to amplify overlapping sections of genomic DNA from the region encoding exons 1–10. After running denatured/reannealed PCR products in the nondenaturing gel matrix (40×20 cm×1 mm) for 21 hr at 20 V/cm, a polymorphism was detected in a 433-bp fragment amplified from three independent PCR reactions by KRp44 (5'-TGTTGAACGATTGGATTAC-3') (sense primer corresponding to nucleotides 456–474) and KRp45 (5'-ATACATATCCACCACTGCT-3') (antisense primer corresponding to nucleotides 702–721). Homozygous-arrested embryos from a heterozygous *h199/e937* parent were used as template for PCR using KRp44 and KRp45. Three independent reaction products were cloned and sequenced to reveal an A → T transversion (nucleotide position

560 in Fig. 4) when compared to sequence obtained from parallel experiments with N2 and *e937* worms as template for PCR.

Northern blot analysis

Mixed stage total N2 *C. elegans* RNA and mixed stage total *bli-4(e937)* RNA was isolated by centrifugation of nematode lysates prepared in guanidinium thiocyanate through CsCl gradients (Chirgwin et al. 1979; Kramer et al. 1985). Poly(A)⁺ RNA was purified using the PolyATtract mRNA isolation system (Promega), according to the manufacturer's conditions. Five micrograms of poly(A)⁺ RNA was denatured in 2.2 M formaldehyde, 50% deionized formamide, and 1× MOPS for 15 min at 60°C. The RNA was fractionated by electrophoresis in an 0.8% agarose gel containing 1× MOPS and 2.2 M formaldehyde (pH 7.0). RNA was transferred to a Zeta-probe membrane (Bio-Rad) and hybridized with random-primed (Feinberg and Vogelstein 1983) ³²P-labeled probes in 0.25 M Na₂HPO₄ (pH 7.2), 7% SDS at 65°C. The membrane was washed in 0.3× SSC, 0.1% SDS, at 65°C. The common probe used was a *Cl*AI-*Acc*II 480-bp fragment corresponding to nucleotides 1609–2089 of the blisterase B cDNA (Fig. 4), which lies just 3' of the protease domain and is common to all of the blisterases. The blisterase C/D-specific probe was a *S*acI-*B*amHI 518-bp fragment corresponding to nucleotides 2239–2757 within the blisterase D sequence shown in Figure 4. The first 275 bp of this fragment are shared by blisterase C; hence, the probe should hybridize to both the C and D transcripts. To standardize RNA levels, the membrane was stripped and rehybridized with a 1.7-kb probe containing genomic sequences encoding the *C. elegans* S-adenosyl homocysteine hydrolase (AHH) gene.

RT-PCR

First-strand cDNA was synthesized from 5 µg of total RNA using a dT_{12–18} primer and the Superscript Preamplification system (GIBCO-BRL). The conditions used were as described by the manufacturer. The primers used for amplification of first-strand cDNA were as follows (see also Fig. 4): KRp6 (5'-CTACTCG-GCTACTCCTGC-3') (nucleotides 2088–2105 in exon 12, common to all of the *bli-4* gene products); KRp49 (5'-TCTAT-GAAGCTGTGAGCAGT-3') (antisense primer corresponding to nucleotides 2209–2228 of the blisterase A-specific 3' exon); KRp50 (5'-ATGGGTACGAGAAGAAGAGT-3') (antisense primer corresponding to nucleotides 2261–2280 of the blisterase B-specific 3' exon), and KRp39 (5'-TAAAGCGCTCGGCT-TTTTG-3') (antisense primer corresponding to nucleotides 2583–2602 of the blisterase C-specific exon 18). Primers OPC18.r and OPC19.c, which are specific for the *ubl* gene (Jones and Candido 1993), were used as an internal positive control. Amplification reactions were performed in which the primer KRp6 was used in combination with one of the antisense primers specific for each BLI-4 isoform, along with the control *ubl* gene primers. Reaction mixtures for PCR contained a 1-µl aliquot of the cDNA template, 50 pmoles of each primer, and 2.5 units of *Taq* DNA polymerase (Promega) in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% Triton X-100, 1.5 mM MgCl₂, and 200 mM dNTPs in a final volume of 25 µl. Reactions were carried out in a Perkin-Elmer Cetus thermal cycler for 32 cycles of denaturation (94°C, 45 sec), annealing (59°C, 30 sec), and extension (72°C, 1 min) followed by extension at 72°C for 7 min.

Determination of the 5' end of *bli-4* was as follows. RT-PCR was performed on poly(A)⁺ RNA from N2 worms. First-strand cDNA was synthesized as above. The resultant cDNA was amplified with KRp10 (5'-ACTCTCTCTTCGGTCGC-3') (antisense primer corresponding to nucleotides 504–521 of exon

3) and a sense primer derived from SL1 (including a *Not*I adapter) (5'-ATAAGAATGCGGCCGCGGTTTAATTACCCA-GTTG-3'). A product of the expected size for KRp10 with SL1 (~500 bp) was reamplified using SL1 and the nested primer KRp11 (5'-GTGTCCTTGTGTTTCCG-3') (antisense primer corresponding to nucleotides 418–436 of exon 3). The resulting product was cloned and sequenced to confirm that *bli-4* is trans-spliced to SL1. A parallel experiment performed with an SL2 trans-splice leader primer failed to amplify *bli-4* sequences. Reaction conditions for PCR were as described above except the annealing temperature for initial amplification on first strand cDNA was 56°C and reamplification was at 58°C.

Note

The accession numbers for the cDNA sequences reported in this paper are L29438, L29439, and L29440.

Acknowledgments

We wish to thank Gary Thomas, Carolyn Brown, David Baillie, Mark Edgley, Marco Marra, and Anna Tinker for helpful discussions concerning the manuscript, Betty Leung for technical assistance with the automated sequencer, Lisa Kadyk and Judith Kimble for the *q508* mutant, Jennifer McDowall for the transgenic strain KR2276, Yuji Kohara for additional cDNA clones and sequence, Ben Williams for the p618 Tc1 primer, and Don Jones and Peter Candido for the *ubl* specific primers. Some nematode strains used in this study were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. This research was supported by grants from the Medical Research Council of Canada and the British Columbia Health Research Foundation to A.M.R. and a Medical Research Council of Canada Studentship to K.P., and by equipment purchased with grants from the British Columbia Health Research Foundation.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Babu, P. 1974. Biochemical genetics of *C. elegans*. *Mol. & Gen. Genet.* **135**: 39–44.
- Baillie, D.L., K.A. Beckenbach, and A.M. Rose. 1985. Cloning within the *unc-43* to *unc-31* interval (linkage group IV) of the *Caenorhabditis elegans* genome by Tc1 linkage selection. *Can. J. Genet. Cytol.* **27**: 457–466.
- Barr, P.J. 1991. Mammalian subtilisins: The long-sought dibasic processing endoproteases. *Cell* **66**: 1–3.
- Barstead, R.J. and R.H. Waterston. 1989. The basal component of the nematode dense-body is vinculin. *J. Biol. Chem.* **264**: 10177–10185.
- Barstead, R.J., L. Kleiman, and R.H. Waterston. 1991. Cloning, sequencing, and mapping of an α -actinin gene from the nematode *Caenorhabditis elegans*. *Cell Motil. Cytoskel.* **20**: 69–78.
- Blumenthal, T. and J. Thomas. 1988. *Cis* and *trans* mRNA splicing in *C. elegans*. *Trends Genet.* **4**: 305–308.
- Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71–94.
- Bresnahan, P.A., R. Leduc, L. Thomas, J. Thorner, H.L. Gibson, A.J. Brake, P.J. Barr, and G. Thomas. 1990. Human *fur* gene encodes a yeast KEX2-like endoprotease that cleaves pro-

- beta-NGF *in vivo*. *J. Cell Biol.* **111**: 2851–2859.
- Chan, S.J., A.A. Oliva Jr., J. LaMendola, A. Grens, H. Bode, and D.F. Steiner. 1992. Conservation of the prohormone convertase gene family in metazoa: Analysis of cDNAs encoding a PC3-like protein from hydra. *Proc. Natl. Acad. Sci.* **89**: 6678–6682.
- Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299.
- Coulson, A., J. Sulston, S. Brenner, and J. Karn. 1986. Towards a physical map of the genome of the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci.* **83**: 7821–7825.
- Coulson, A., R. Waterston, J. Kiff, J. Sulston, and Y. Kohara. 1988. Genome linking with yeast artificial chromosomes. *Nature* **335**: 184–186.
- Docherty, K. and D.F. Steiner. 1982. Post-translational proteolysis in polypeptide hormone biosynthesis. *Annu. Rev. Physiol.* **44**: 625–638.
- Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137**: 266–267.
- Fire, A., S.W. Harrison, and D. Dixon. 1990. A modular set of *lacZ* fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **113**: 189–198.
- Fodor, A. and P. Deak. 1985. The isolation and genetic analysis of a *Caenorhabditis elegans* translocation (*szT1*) strain bearing an X-chromosome balancer. *J. Genet.* **64**: 143–157.
- Fuller, R.S., R.E. Sterner, and J. Thorner. 1988. Enzymes required for yeast prohormone processing. *Annu. Rev. Physiol.* **50**: 345–362.
- Fuller, R.S., A. Brake, and J. Thorner. 1989a. Yeast prohormone processing enzyme (KEX2 gene product) is a Ca^{2+} -dependent serine protease. *Proc. Natl. Acad. Sci.* **86**: 1434–1438.
- Fuller, R.S., A.J. Brake, and J. Thorner. 1989b. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science* **246**: 482–486.
- Fuller, R.S., C. Brenner, P. Gluschkof, and R.S. Wilcox. 1991. The yeast prohormone-processing KEX2 protease, an enzyme with specificity for paired basic residues. In *Advances in life sciences: Methods in protein sequence analysis* (ed. Jornvall, H., J.-O. Hoog, and A.-M. Gustavsson), pp. 205–214. Birhauser, Berlin, Germany.
- Gluschkof, P. and R.S. Fuller. 1994. A C-terminal domain conserved in precursor processing proteases is required for intramolecular N-terminal maturation of pro-Kex2 protease. *EMBO J.* **13**: 2280–2288.
- Gómez-Saladín, E., D.L. Wilson, and I.M. Dickerson. 1994. Isolation and *in situ* localization of a cDNA encoding a Kex2-like prohormone convertase in the nematode *Caenorhabditis elegans*. *Cell. Mol. Neurobiol.* **14**: 9–25.
- Hayflick, J.S., W.J. Wolfgang, M.A. Forte, and G. Thomas. 1992. A unique Kex-2 like endoprotease from *Drosophila melanogaster* is expressed in the central nervous system during early embryogenesis. *J. Neurosci.* **12**: 705–717.
- Henikoff, S. 1984. Unidirectional digestion with exonuclease III in DNA sequence analysis. *Gene* **28**: 351–259.
- Horvitz, H.R., S. Brenner, J. Hodgkin, and R. Herman. 1979. A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. *Mol. & Gen. Genet.* **175**: 129–133.
- Hosaka, M., M. Nagahama, W.-S. Kim, T. Watanabe, K. Hatuszawa, J. Ikemizu, K. Murakami, and K. Nakayama. 1991. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalysed by furin within the constitutive secretory pathway. *J. Biol. Chem.* **266**: 12127–12130.
- Howell, A.M., S.G. Gilmore, R.A. Mancebo, and A.M. Rose. 1987. Genetic analysis of a large autosomal region in *Caenorhabditis elegans* by the use of a free duplication. *Genet. Res.* **49**: 207–213.
- Ikemura, H. and M. Inouye. 1988. *In vitro* processing of pro-subtilisin produced in *Escherichia coli*. *J. Biol. Chem.* **263**: 12959–12963.
- Jones, D. and E.P.M. Candido. 1993. Novel ubiquitin-like ribosomal protein fusion genes from the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*. *J. Biol. Chem.* **268**: 19545–19551.
- Kiefer, M.C., J.E. Tucker, R. Joh, K.E. Landsberg, D. Saltman, and P.J. Barr. 1991. Identification of a second human subtilisin-like protease gene in the *fes/fps* region of chromosome 15. *DNA Cell Biol.* **10**: 757–769.
- Kramer, J.M. and J.J. Johnson. 1993. Analysis of mutations in the *sqt-1* and *rol-6* collagen genes of *Caenorhabditis elegans*. *Genetics* **135**: 1035–1045.
- Kramer, J.M., G.N. Cox, and D. Hirsh. 1985. Expression of the *Caenorhabditis elegans* collagen genes *col-1* and *col-2* is developmentally regulated. *J. Biol. Chem.* **260**: 1945–1951.
- Kramer, J.M., R.P. French, E.-C. Park, and J.J. Johnson. 1990. The *Caenorhabditis elegans* *rol-6* gene, which interacts with the *sqt-1* collagen gene to determine organismal morphology, encodes a collagen. *Mol. Cell Biol.* **10**: 2081–2089.
- Krause, M. and D. Hirsh. 1987. A trans-spliced leader sequence on actin mRNA in *Caenorhabditis elegans*. *Cell* **49**: 735–761.
- Kyte, J. and R.F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**: 105–132.
- Leduc, R., S.S. Molloy, B.A. Thorne, and G. Thomas. 1992. Activation of human furin precursor processing endoprotease occurs by an intramolecular autoproteolytic cleavage. *J. Biol. Chem.* **267**: 14304–14308.
- Liebowitz, M.J. and R.W. Wickner. 1976. A chromosomal gene required for killer plasmid expression, mating and sporulation in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **73**: 2061–2065.
- Lusson, J., D. Vieau, J. Hamelin, R. Day, M. Chrétien, and N.G. Seidah. 1993. cDNA structure of the mouse and rat subtilisin/kexin-like PC5: A candidate proprotein convertase expressed in endocrine and nonendocrine cells. *Proc. Natl. Acad. Sci.* **90**: 6691–6695.
- McKim, K.S., A.M. Howell, and A.M. Rose. 1988. The effects of translocations on recombination frequency in *Caenorhabditis elegans*. *Genetics* **120**: 987–1001.
- Mello, C.C., J.M. Kramer, D. Stinchcomb, and V. Ambros. 1991. Efficient gene transfer in *C. elegans*: Extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**: 3959–3970.
- Mizuno, K., T. Nakamura, T. Ohshima, S. Tanaka, and H. Matsuo. 1988. Yeast KEX2 gene encodes an endopeptidase homologous to subtilisin-like serine proteases. *Biochem. Biophys. Res. Commun.* **156**: 246–254.
- Molloy, S.S., P.A. Bresnahan, S.H. Leppla, K.R. Klimpel, and G. Thomas. 1992. Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. *J. Biol. Chem.* **267**: 16396–16402.
- Molloy, S.S., L. Thomas, J.K. VanSlyke, P.E. Stenberg, and G. Thomas. 1994. Intracellular trafficking and activation of the furin proprotein convertase: Localization to the TGN and recycling from the cell surface. *EMBO J.* **13**: 18–33.
- Mori, I., D.G. Moerman, and R.H. Waterston. 1988. Analysis of a mutator activity necessary for germline transposition and excision of Tc1 transposable elements in *Caenorhabditis el-*

- egans. Genetics* **120**: 397–407.
- Nakayama, K., W.-S. Kim, S. Torii, M. Hosaka, T. Nakagawa, J. Ikemizu, T. Baba, and K. Murakami. 1992. Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease. *J. Biol. Chem.* **267**: 5897–5900.
- Nakagawa, T., M. Hosaka, S. Torii, T. Watanabe, K. Murakami, and K. Nakayama. 1993. Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family: Its striking structural similarity to PACE4. *J. Biochem.* **113**: 132–135.
- Park, E.-C. and H.R. Horvitz. 1986. Mutations with dominant effects on the behavior and morphology of the nematode *C. elegans*. *Genetics* **113**: 821–852.
- Peters, K., J. McDowall, and A.M. Rose. 1991. Mutations in the *bli-4 (I)* locus of *Caenorhabditis elegans* disrupt both adult cuticle and early larval development. *Genetics* **129**: 95–102.
- Pearson, W.R. and D.J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci.* **85**: 2444–2448.
- Power, S.D., R.M. Adams, and J.A. Wells. 1986. Secretion and autoproteolytic maturation of subtilisin. *Proc. Natl. Acad. Sci.* **83**: 3096–3100.
- Priess, J.R. and D.I. Hirsh. 1986. *Caenorhabditis elegans* morphogenesis: The role of the cytoskeleton in elongation of the embryo. *Dev. Biol.* **117**: 156–173.
- Roebroek, A.J.M., J.A. Schalken, J.A.M. Leunissen, C. Onnekink, H.P.J. Bloemers, and W.J.M. Van de Ven. 1986. Evolutionary conserved close linkage of the *c-fes/fps* proto-oncogene and genetic sequences encoding a receptor-like protein. *EMBO J.* **5**: 2197–2202.
- Roebroek, A.J.M., J.W.M. Creemers, I.G.L. Pauli, U. Kurzik-Dumke, M. Rentrop, E.A.F. Gateff, J.A.M. Leunissen, and W.J.M. Van de Ven. 1992. Cloning and functional expression of Dfurin2, a subtilisin-like proprotein processing enzyme of *Drosophila melanogaster* with multiple repeats of a cysteine motif. *J. Biol. Chem.* **267**: 17208–17215.
- Roebroek, A.J.M., J.W.M. Creemers, I.G.L. Pauli, T. Bogaert, and W.J.M. Van de Ven. 1994. Generation of structural and functional diversity in furin-like proteins in *Drosophila melanogaster* by alternative splicing of the *Dfur1* gene. *EMBO J.* **12**: 1853–1870.
- Rose, A.M. and D.L. Baillie. 1980. Genetic organization of the region around *unc-15 (I)*, a gene affecting paramyosin in *Caenorhabditis elegans*. *Genetics* **96**: 639–648.
- Rose, A.M., D.L. Baillie, and J. Curran. 1984. Meiotic pairing behavior of two free duplications of linkage group I of *Caenorhabditis elegans*. *Mol. & Gen. Genet.* **195**: 52–56.
- Ruoslahti, E. and M.D. Pierschbacher. 1986. Arg-Gly-Asp: A versatile cell recognition signal. *Cell* **44**: 517–518.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Seidah, N.G., L. Gaspar, P. Mion, M. Marcinkiewicz, M. Mbikay, and M. Chrétien. 1990. cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: Tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA Cell Biol.* **9**: 415–424.
- Seidah, N.G., M. Marcinkiewicz, S. Benjannet, L. Gaspar, G. Beaubien, M.G. Mattei, C. Lazure, M. Mbikay, and M. Chrétien. 1991. Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, furin and Kex2: Distinct chromosomal localization and messenger RNA distribution in the brain and pituitary compared to PC2. *Mol. Endocrinol.* **5**: 111–122.
- Seidah, N.G. and M. Chrétien. 1992. Proprotein and prohormone convertases of the subtilisin family. *Trends Endocrinol. Metab.* **3**: 133–140.
- Seidah, N.G., R. Day, J. Hamelin, A. Gaspar, M.W. Collard, and M. Chrétien. 1992. Testicular expression of PC4 in the rat: Molecular diversity of a novel germ cell-specific kex2/subtilisin-like proprotein convertase. *Mol. Endocrinol.* **6**: 1559–1570.
- Smeekens, S.P. and D.F. Steiner. 1990. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *J. Biol. Chem.* **265**: 2997–3000.
- Smeekens, S.P., A.S. Avruch, J. LaMendola, S.J. Chan, and D.F. Steiner. 1991. Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. *Proc. Natl. Acad. Sci.* **88**: 340–344.
- Starr, T., A.M. Howell, J. McDowall, K. Peters, and A.M. Rose. 1989. Identification of DNA probes within the linkage group I gene cluster of *Caenorhabditis elegans*. *Genome* **32**: 365–372.
- Thomas, G., B.A. Thorne, and D.E. Hurby. 1988. Gene transfer techniques to study neuropeptide processing. *Annu. Rev. Physiol.* **50**: 323–332.
- van den Ouweland, A.M., H.L. van Duijnhoven, G.D. Keizer, L.C. Dorssers, and W.J.M. Van de Ven. 1990. Structural homology between the human *fur* gene product and the subtilisin-like protease encoded by yeast KEX2. *Nucleic Acids Res.* **18**: 664.
- Van de Ven, W.J.M., J. Voorberg, R. Fontijn, H. Pannekoek, A.M.W. van den Ouweland, H.L.P. van Duijnhoven, A.J.M. Roebroek, and R.J. Siezen. 1990. Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. *Mol. Biol. Rep.* **14**: 265–275.
- Waterston, R., C. Martin, M. Craxton, C. Huynh, A. Coulson, L. Hillier, R. Durbin, P. Green, R. Shownkeen, N. Halloran, M. Metzstein, T. Hawkins, R. Wilson, M. Berks, Z. Du, K. Thomas, J. Thierry-Mieg, and J. Sulston. 1992. A survey of expressed genes in *Caenorhabditis elegans*. *Nature Genet.* **1**: 114–123.
- Wickner, R.W. and M.J. Liebowitz. 1976. Two chromosomal genes required for killing expression in killer strains of *Saccharomyces cerevisiae*. *Genetics* **82**: 429–442.
- Wise, R.J., P.J. Barr, P.A. Wong, M.C. Kiefer, A.J. Brake, and R.J. Kaufman. 1990. Expression of a human proprotein processing enzyme: Correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proc. Natl. Acad. Sci.* **87**: 9378–9382.
- Yandell, M.D., L.G. Edgar, and W.B. Wood. 1994. Trimethylpsoralen induces small deletion mutations in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci.* **91**: 1381–1385.
- Yang, J. and J.M. Kramer. 1994. *In vitro* mutagenesis of *Caenorhabditis elegans* cuticle collagens identifies a potential subtilisin-like protease cleavage site and demonstrates that carboxyl domain disulphide bonding is required for normal function but not assembly. *Mol. Cell Biol.* **14**: 2722–2730.

The *bli-4* locus of *Caenorhabditis elegans* encodes structurally distinct kex2/subtilisin-like endoproteases essential for early development and adult morphology.

C Thacker, K Peters, M Srayko, et al.

Genes Dev. 1995, **9**:

Access the most recent version at doi:[10.1101/gad.9.8.956](https://doi.org/10.1101/gad.9.8.956)

References

This article cites 70 articles, 31 of which can be accessed free at:
<http://genesdev.cshlp.org/content/9/8/956.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).