A Unique Kex2-like Endoprotease from *Drosophila melanogaster* Is Expressed in the Central Nervous System during Early Embryogenesis

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Complementary DNA sequences were cloned from a *Drosophila* library encoding a 1101 amino acid polypeptide that we have named dKLIP-1. The deduced protein is structurally similar to the yeast KEX2 prohormone endoprotease including the conserved Asp, His, and Ser catalytic triad residues characteristic of the subtilisin family. When coexpressed *in vivo* with pro- β -NGF, dKLIP-1 greatly enhanced the endoproteolytic conversion of the precursor to mature β -NGF by cleavage at a -Lys-Arg- doublet. In adults, dKLIP-1 transcripts were detected in cortical regions of the CNS and fat body. Most striking, however, was the high level of maternal transcripts deposited into developing oocytes. The temporal and spatial expression of dKLIP-1 mRNAs during embryonic development indicates a potential role for this novel Kex2p-like endoprotease in early embryogenesis and neurogenesis.

Endoproteolysis of precursor proteins is a common step required for the synthesis of biologically active proteins and peptides in all eukaryotes examined, including yeast (Fuller et al., 1988), invertebrate (Scheller et al., 1983), and mammalian cells (Douglass et al., 1984). Typically, the cleavage occurs at the carboxyl side of pairs of basic amino acid sequences (especially -Lys-Arg- and -Arg-Arg-). Excision of bioactive peptides and proteins by cleavage at doublets of basic amino acids was first proposed from studies of proinsulin (Steiner et al., 1967; Chance et al., 1968) and the ACTH/ β -endorphin precursor proopiomelanocortin (POMC) (Chretien and Li, 1967). Subsequent studies have revealed a broad spectrum of precursor proteins that require endoproteolysis at pairs of basic amino acids to yield mature peptides, including serum factors (Bentley et al., 1986),

viral envelope proteins (McCune et al., 1988), growth factors (Mason et al., 1985), and receptors (Yoshimasa et al., 1988).

A number of activities capable of cleaving at paired basic residues *in vitro* have been proposed as candidates for authentic mammalian precursor processing endoproteases (reviewed in Sossin et al., 1989); however, none have been shown to be a bona fide precursor cleaving endoprotease *in vivo*. In contrast, genetic and biochemical studies unequivocally identified the gene in *Saccharomyces cerevisiae* required for excision of the α -factor mating hormone from its precursor (Leibowitz and Wickner, 1976; Julius et al., 1984). This locus, the KEX2 gene, encodes a subtilisin-like, membrane-bound, calcium-dependent, serine endoprotease (Kex2p) specific for cleaving on the carboxyl side of pairs of basic residues (-Lys-Arg- and -Arg-Arg-) (Fuller et al., 1989a; Mizuno et al., 1989).

Several lines of evidence suggest that precursor processing endoproteases in higher eukaryotes are functionally and, hence, structurally similar to Kex2p. The mammalian precursor proalbumin, for example, is efficiently converted *in vitro* to albumin by Kex2p, with cleavage at a paired basic amino acid sequence (-Arg-Arg-) (Bathurst et al., 1987). Furthermore, coexpression of the KEX2 gene with mouse POMC in BSC-40 cells (a line incapable of processing this peptide precursor) resulted in efficient proteolysis, on the carboxyl side of basic amino acid pairs, producing authentic pituitary peptides (Thomas et al., 1988a).

Recently, three DNA sequences, *fur*, PC2, and PC3 (also called PC1) have been reported, which share significant sequence identity with the KEX2 gene sequence (Fig. 1) (Fuller et al., 1989b; Seidah et al., 1990, 1991; Smeekens and Steiner, 1990; van den Ouweland et al., 1990; Wise et al., 1990; Smeekens et al., 1991). RNA hybridization studies demonstrate that the *fur* gene is ubiquitously expressed in a variety of tissues and all cell lines examined (Schalken et al., 1987; Bresnahan et al., 1990; Hattuzawa et al., 1990). In contrast, expression of PC2 and PC3 is restricted to neuroendocrine tissues. Gene transfer studies demonstrated that the *fur* gene product, furin, as well as PC2 and PC3, are Kex2p-like endoproteases capable of efficiently processing precursor proteins at pairs of basic amino acids (Bresnahan et al., 1990; van de Ven et al., 1990; Wise et al., 1990; Benjannet et al., 1991; Thomas et al., 1991). Like Kex2p, furin is a Golgi membrane-localized endoprotease that can efficiently process pro- β -NGF when coexpressed in mammalian cells (Bresnahan et al., 1990). Comparable results were reported on the enhanced processing of pro-von Willebrand factor by furin (van de Ven et al., 1990; Wise et al., 1990). In contrast, the neuroendocrine-specific endoproteases PC2 and PC3 cleave

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Kex2p ...171AAIVDDGLDYEN...212YHGTRCAGEI...382GGTSAAPLAAGV
hfurin...149VSILDDGIEKNH...193RHGTRCAGEV...365TGTSAAPLAAGI
hPC2 ...138IGIMDDGIDYLH...207SHGTRCAGEV...381SGTSAAPLAAGV
Consensus --I-DDG----- -HGTRCAGE- -GTSA-AP-AAG-

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Figure 1. Alignment of the amino acid sequences surrounding the catalytic triad residues in Kex2p, human furin (*hfurin*), and human PC2 (*hPC2*). The coding sequences surrounding the His and Ser residues showed greatest sequence identity and therefore were used for the design of PCR primer pools. The amplified ~542 bp product predicted from PCR experiments was determined by adding the lengths of the primers (24 and 32 bases) to the sequence in Kex2p that separated their 3' ends (486 bases). Computer-based alignment of polypeptide sequences for Kex2p (Mizuno, 1988), hfurin (van de Ouweland et al., 1990), and hPC2 (Smeekens and Steiner, 1990) was done using the BESTFIT program (Devereux et al., 1984). Nonidentical residues are shown by dashes in the consensus (bottom) line. The asterisks denote asparagine, histidine, and serine residues of the catalytic triad.

mouse POMC correctly at doublets of basic amino acids into sets of pituitary peptides (Benjannet et al., 1991; Thomas et al., 1991).

The identification of multiple Kex2p-like endoproteases expressed in a wide variety of tissues and cell lines suggests that each homolog catalyzes the maturation of several bioactive peptides and proteins. In yeast, genetic tools have been key to understanding the role of Kex2p. Unlike yeast, however, mammalian systems are generally intractable to classical genetic analyses. In contrast, *Drosophila* provides an excellent system to examine the role of this newly identified group of metazoan precursor endoproteases. With *Drosophila*, the tools of classical and molecular genetics can be applied systematically in an organism in which each of the developmental stages is well described and easily accessible (Rubin, 1988). To this end, we report here the cloning, tissue and developmental expression, and endoproteolytic activity of a *Drosophila* Kex2p-like endoprotease (dKLIP-1).

Materials and Methods

Fly stocks and reagents. Oregon R stocks were maintained at 25°C on 12 hr light/12 hr dark schedules on a standard diet (Roberts, 1986). Restriction enzymes T7 RNA polymerase, SP6 RNA polymerase, and Taq DNA polymerase were purchased from Bethesda Research Labs, Inc. (BRL). Oligonucleotides were purchased from Oligos Etc. Inc. The adult fly head gt11 library was a gift from P. Salvaterra (City of Hope, Duarte, CA). Oligo-dT cellulose was purchased from Boehringer Mannheim Biochemicals, Inc.

Cell culture. BSC-40 cells, an African green monkey kidney epithelial cell line, and HeLa cells, a human cervical epithelial cell line, were cultured in minimum essential medium (MEM) (GIBCO Laboratories) containing 10% heat-inactivated fetal bovine serum (HyClone Laboratories).

Polymerase chain reaction and library screening. Two micrograms of λ -DNA from the library used below containing oligo-dT-primed adult head-specific cDNAs were amplified with 100 pm each of two oligonucleotide pools: pool 1, 5'-CA(C/T)GG(C/A/T)AC(C/G)CG(A/C/G)TG(T/C)GC(C/G)GG(A/T/C)GA(G/A); pool 2, 5'-CC(G/A)GC(G/A)GCCA(G/A)GGG(G/A)GCGG(A/C)(G/A)GC(G/A/T/C)GAGG-T(G/T)CC. Twenty-five microliter PCR reactions were performed using Taq polymerase with temperature and time parameters of 95°C, 1 min, 55°C, 1 min, 74°C, 1 min for 30 cycles. The product was agarose gel purified and subcloned into pGEM7Zf+ (Promega Corp.). One hundred nanograms of PCR product were radio labeled by the random primer method (Feinberg and Vogelstein, 1983) and used to screen 5×10^5 plaques of the λ gt11 fly head library. Hybridization was performed in 50% formamide, 1% SDS, 1 M NaCl, 10% dextran SO_4 , and 100 μ g/ml sheared, denatured salmon sperm DNA at 37°C for 18 hr. Filters were washed briefly in $2 \times$ SSC (saline-sodium citrate) at room temperature and then in 0.1% SDS, 0.1 $\times$ SSC at 65°C for 60 min. Positively hybridizing clones were purified and two phage isolates (1L and 3) were chosen for further study. The composite dKLIP-1 cDNA (see Fig. 3A caption) was ligated into the EcoRI site of pGEM7Zf+, and the resulting plasmid (pGEM7Zf+:dKLIP-1) was used for later studies.

DNA sequencing. Fragments of λ -phages 3 and 1L were subcloned into M13mp18 or mp19 vectors (Yanisch-Perron et al., 1985) and sequenced using the dideoxynucleotide chain terminator method (Tabor and Richardson, 1987) and Sequenase v2 (U.S. Biochemical). Reactions were run on 5% denaturing polyacrylamide wedge gels (Chen and Seeburg, 1985). Sequences of overlapping restriction fragments were determined from both DNA strands.

Computer analyses. DNA sequences were compiled and analyzed using the Genetics Computer Group Sequence Analysis Software Package, version 6.2, 1990 (Devereux et al., 1984) or the Intelligenetics, Inc. programs. The deduced protein sequence for dKLIP-1 was compared to the Kex2p homologs using the BESTFIT and GENALIGN programs. Hydrophobicity analysis was performed using the PEPLOT program.

RNA isolation and Northern blot analyses. Fly heads were separated from bodies and stored at -70°C until use. Staged embryos were collected on apple juice agar plates and maintained at 25°C until harvest. One gram of whole flies or parts was crushed in liquid N_2 . Total RNA was isolated as described (Cathala et al., 1983). Ten micrograms of each polyA⁺-selected RNA sample were fractionated by electrophoresis in 1% agarose (FMC), 6% formaldehyde, 0.02 M HEPES, 0.001 M EDTA, pH 7.8 gels and transferred to a nylon membrane (Hybond-N, Amersham). Hybridizations were carried out in 50% formamide, $5 \times$ SSC, 0.025 M NaPO₄, $5 \times$ Denhardt's solution, 0.005 M EDTA, 0.1% sodium pyrophosphate, 1% SDS, and 100 μ g/ml sheared, denatured salmon sperm DNA at 37°C for 18 hr. Blots were washed in $2 \times$ SSC at room temperature and 0.1 $\times$ SSC, 0.1% SDS at 65°C for 60 min. Filters were then exposed to X-OMAT AR film (Kodak).

In vitro expression. RNA was synthesized *in vitro* essentially as described (Krieg and Melton, 1987). Briefly, 10 μ g of pGEM7Zf+:dKLIP-1 were linearized with XhoI endonuclease. Fifty microliter RNA transcription reactions contained 1 μ g of linearized plasmid DNA in $1 \times$ SP6 buffer; 0.01 M dithiothreitol DTT; 50 μ g/ml bovine serum albumin; 1 U placental RNase inhibitor (Promega); 250 μ M each ATP, CTP, and UTP; 50 μ M GTP; 250 μ M GpppG; and 50 U of SP6 RNA polymerase (BRL). Incubations were performed at 37°C for 5 hr. RNA was extracted twice with phenol:chloroform, ethanol precipitated, and resuspended in 50 μ l H₂O. Fifty microliter *in vitro* translation reactions containing *in vitro* transcribed RNA, rabbit reticulocyte lysate (Promega) and ³H-leucine were set up as described by the manufacturer. After 30 min at 30°C, 10% of the translation reaction was run on a 7% SDS-PAGE (Laemmli, 1970), which was then processed for fluorography (Amplify, Amersham). The dried gel was exposed to X-OMAT film for 16 hr at -70°C. For translation/translocation studies, canine pancreatic microsomes (Promega) were added to the reticulocyte reactions. Protease-digested reactions were adjusted to 0.01 M CaCl₂ and 0.1 mg/ml proteinase K, and detergent-disrupted reactions were brought to 0.1% Triton X-100. Following a 45 min incubation on ice, the digestions were terminated by adjusting to 2 mM phenylmethylsulfonyl fluoride and analyzed by SDS-PAGE as described above.

Vaccinia virus. Vaccinia virus (VV) strain WR was used and infections were performed as described (Thomas et al., 1988b). Recombinant virus carrying the dKLIP-1 cDNA was constructed as follows. The 3.8 kilobase (kb) dKLIP-1 cDNA was ligated directionally into pZVneo. The plasmid pZVneo contains the vaccinia thymidine kinase gene interrupted by a VV expression/selection cassette putting the expression of dKLIP-1 under the control of the constitutive p7.5 VV promoter. The Tn5 aminoglycoside transferase gene driven by the VV p11 promoter acts as a selectable marker by conferring neomycin resistance. The resulting plasmid (pZVneo:dKLIP-1) was used to introduce the cDNA

and plasmid flanking sequences into vaccinia virus by homologous recombination (Hruby et al., 1986). Purified isolates of VV:dKLIP-1 were used for the production of viral stocks.

Coexpression studies of dKLIP-1 with murine pro- β -NGF were performed essentially as described (Bresnahan et al., 1990). Briefly, parallel wells of BSC-40 cells were infected with VV:mNGF (m.o.i. = 2) and either VV:WT or VV:dKLIP-1 (m.o.i. = 5; total m.o.i. = 7). At 19 hr postinfection, the cells were starved for methionine for 30 min and then pulsed with 100 μ Ci 35 S-Met, Cys (Express Label, New England Nuclear-Du Pont). After 3 hr, the medium was harvested and secreted products were immunoprecipitated by overnight incubation with a rabbit anti-mouse β -NGF serum (gift of S. Ojeda), following which immune complexes were collected using Protein-A Sepharose beads (Zymed Labs). The beads were washed twice with 1% NP40/1 M NaCl and twice in RIPA buffer (Harlow and Lane, 1988). Immune complexes were analyzed by SDS-PAGE in 12.5% gels (Laemmli, 1970). Fixed gels were then impregnated with fluor (Amplify, Amersham) and exposed to x-ray film.

In situ hybridizations. Sense and antisense RNA probes were generated by using the SP6 or T7 promoters of pGEM7Zf+:dKLIP-1 using α - 35 S-thio-UTP. Probes were hybridized to 8 μ m tissue sections that had been mounted on microscope slides and fixed with 4% formaldehyde, then treated in 0.2 M HCl for 20 min, $2\times$ SSC for 30 min at 60°C, proteinase K at 37°C for 15 min, 4% formaldehyde for 20 min, and then acetylated and dried (Hafen and Levine, 1986). After overnight hybridization at 50°C, the sections were rinsed briefly in $4\times$ SSC and then incubated for 15 min at 60°C in 50% formamide, 0.3 M NaCl, 0.03 M Tris, pH 7.5, 1 mM EDTA, and 10 mM DTT. The slides were then washed in 4 liters of $2\times$ SSC at 25°C for 30 min, $0.1\times$ SSC (55°C, 10 min) and $0.1\times$ SSC (25°C, 10 min). After drying the slides were coated with photographic emulsion (Kodak NTB2).

Embryos were collected and prepared by previously described methods (Mitchison and Sedat, 1983). Sense and antisense transcripts were synthesized in the presence of digoxigenin-11-UTP (Boehringer Mannheim). Following RNase-free DNase treatment, the transcripts were subjected to limited hydrolysis in 0.1 M carbonate buffer, pH 10.2 (Angerer and Angerer, 1981), yielding fragments of 100–125 nucleotides as determined by gel electrophoresis. Whole-mount embryo hybridization and detection were done as previously described (Tautz and Pfeifle, 1989). Embryonic stages are as described by Campos-Ortega and Hartenstein (1985).

Results

Cloning and characterization of the dKLIP-1 cDNA

A strategy for the identification of cDNAs encoding Kex2p-like endoproteases from *Drosophila* was developed by examining an alignment of the amino acid sequences of human furin (hfurin) (van de Ouweland, 1990), human PC2 (hPC2) (Smeekens and Steiner, 1990), and yeast Kex2p (Mizuno et al., 1988). Significant identity exists in the amino acid sequences that surround the residues forming the catalytic triad (Asp, His, and Ser), with the greatest sequence conservation around the histidine and serine residues (Fig. 1). Based on this conservation and on *Drosophila* codon bias data (Wada et al., 1990), two degenerate oligonucleotide primer pools were designed for use in PCR (Sai-ki et al., 1988). The first primer pool was a sense 24mer that encoded eight amino acids beginning with the active-site histidine residue. The second primer pool was an antisense 32mer corresponding to the coding region for the 11 amino acids surrounding the active-site serine residue. A subset of these oligonucleotide primers was expected to anneal to *Drosophila* Kex2p-like cDNA sequences, producing an amplified product of ~542 base pairs (bp). Indeed, PCR experiments using template DNA prepared from a *Drosophila* adult head cDNA library resulted in the isolation of an ~550 bp product. DNA sequence analysis demonstrated the presence of an open reading frame (ORF) that shared significant homology to the predicted region of the Kex2p-like endoproteases.

Two independent phage clones were isolated from the adult

head library using the labeled PCR product as a probe (Fig. 2A). Clones 3 and 1L contained cDNA inserts of 3.5 and 2.8 kb, respectively. Clone 1L contained an ORF with a methionine residue at nucleotide (nt) 232 and extended to the 3' end of the sequence. The nucleotide sequence surrounding this putative initiation codon (Fig. 2B, nt 225–235) was identical to the *Drosophila* consensus translation initiation site [ANN(C/A)A(A/C)(A/C)ATGN; Cavener, 1987]. In addition, this methionine residue was preceded by several in frame termination codons. Clone 3 initiated at nucleotide 240 of clone 1L and continued to a termination codon at position 3535. The protein product predicted from the two overlapping cDNA sequences was 1101 amino acids in length. A composite cDNA (hereafter referred to as the dKLIP-1 cDNA) of 3.78 kilobase pairs containing the complete ORF for dKLIP-1 was created (Fig. 2A).

The dKLIP-1 cDNA contained several interesting features. Proximal to the putative initiator methionine was an opa element (Fig. 2B, nt 258–347), a CAX_n trinucleotide repetitive sequence found in several developmentally regulated mRNAs (Wharton et al., 1985; Grabowski et al., 1991). The 245 nucleotide 3' untranslated region (UTR) contained several AUUUA repeats (Fig. 2B, nt 3570 and 3744), a motif that affects mRNA instability (Shaw and Kamen, 1986), and a canonical polyadenylation addition sequence (AAUAAA, nt 3755; Proudfoot and Brownlee, 1976) 19 bp from a polyA sequence.

The location of the gene encoding dKLIP-1 was identified by *in situ* hybridization of a biotin-labeled dKLIP-1 cDNA probe to *Drosophila* salivary gland chromosomes. Hybridization was observed on the right arm of chromosome 3 at position 96D1-2 (data not shown).

Characterization of the predicted dKLIP-1 polypeptide

Hydrophobicity analyses of the Kex2p-like endoproteases indicated that each has an amino terminal hydrophobic sequence (signal sequence) (Fig. 3B). In the case of Kex2p, this sequence has been shown to target the protein into the secretory pathway, where it becomes glycosylated (Fuller et al., 1986, 1989b). A similar analysis of the predicted protein sequence of dKLIP-1 using a hydrophobicity algorithm (Kyte and Doolittle, 1982) showed that, unlike the other Kex2p-related proteins, dKLIP-1 lacks a signal sequence at its amino terminus. The hydrophobic domain nearest to the amino terminus is situated between residues 118 and 151 (Fig. 3A). This analysis also indicated the presence of a second hydrophobic region near the carboxyl terminus in dKLIP-1 (residues 1006–1036), similar to Kex2p and furin, that is a putative transmembrane domain.

The percentages of overall amino acid sequence conservation between dKLIP-1 and other members of this proteinase family are shown in Table 1. The most conserved region of dKLIP-1 is the subtilisin-like catalytic domain (Fig. 3C), suggesting functional and structural homology with other members of this family. In addition to highly conserved catalytic domains, Kex2p and its mammalian homologs have similar sizes, ranging from 638 residues for hPC2 to 814 residues for Kex2p (Fig. 3B). In contrast, the predicted length of dKLIP-1, 1101 residues, is significantly longer than any reported Kex2p-related sequence. Alignment of the catalytic domains of each of the Kex2p-like homologs indicates that the longer size of dKLIP-1 is due to an extended amino-terminus. The alignment also results in a shared positioning of two sets of paired basic residues in dKLIP-1 (–Lys²⁷⁶–Arg²⁷⁷– and –Lys³⁰⁸–Arg³⁰⁹–) with a small cluster of paired basic amino acids in the other homologs that could be

B

GACCAACAATAATTTAAATTGAGATTTTCATATATCAAAGTGTACAGGCCTCATTTTGCGAGTTATCAGCTCGAAGTGGGTGAAGTGCTATCCTGGACTTTTCGTTTTAGTCAACC 120
CTTGGCCCTCATTAACATCCACTTAATTGAAATAGAACATTTGCATCTCAGCCTCACAAACCAATATCCCAGAACCTGCACAAAACATATTGAAAACTTCAACCCACAATAAAGAAAC 240
MetLysAsn
GACGCTGCTCGGATGGAGCAGGCCCACTAGCAACACCACTTAACAGCAGCAGCAGTAGAAGCGATAGCAACGACCTCATYAGCAACAGCAAGCAAAAGCAAAATTAATAGCTCGA 360
AspValValArgTrpSerArgGlnProThrSerAsnThrThrAsnSer 480
CAATTAGGGTCAAAATGCTGCCAGAAGTTGTCAGCAAAAGTCCTCTGTGGCAACAACTAGAAGTAGAACACAAACAACTATTGAATGTGATATAGGAAATTTCAATTTTCGATTGCAAT 600
GlnLeuGlySerAsnAlaAlaArgSerCysGlnGlnArgSerSerValAlaThrThrLeuGluAspGluGlnGlnThrIleIleGluCysAspIleGlyAsnPheAsnPheAspCysAsn 820
TTATTCAAAAGTAGTTTTTAACGCAACACAAACAGAAAGTAGTGCAAGAGCAGCAGCAAAAGTAAAGAACACAAAGTAGACCCCTAGCGAAAACGAAACGCGGTGTTCTATTAGCT 1000
LeuPheLysThrThrPheLeuThrGlnHisLysGlnLysArgSerGlyLysSer 1220
CTGCAATTTAGTGCCGCTATTTTTTATGTAATAATTAATGTCGGTTCTGTGGCCGGAAGTGTGCAATCTGCGGCATCATCGGCA GCGGCGCTCATCGCCGAGCTCCATCCTCTGCCGCC 1240
LeuGlnPheSerAlaValValPheLeuCysAsnIleAsnValIGlYPheValAlaGlySerValAlaThrAlaAlaSerSerSerSerSerSerSerSerSerSerSerSerSerSerSerSer 1460
TCATCCCCGCCACAGTTGCTGTACCCAGCCGCCGCCACCTTCCTCGGCACCTCAAAGTGGATCCAAATGGTCAGTCACCACTGCTGCCGCCCTACGTTCTCGATTATGAGACGGGGGC 1680
SerSerProProThrValAlaValProProProProProProSerSerAlaLeuLysValAspProAsnGlyGlnSerProValLeuProProTyThrValLeuAspTyrGluThrGlyGly 2000
AAGGCCAAGCTAACGCCAAACAAATGCCAAGTTCGGCCCAATCGGGCAGTTCGGGGAGCAATAACACACACATCGTCGGACCATATACCCACACCTGGCGGGTGCACATACCAACGGCGAT 2240
LysAlaLysLeuThrProProAsnGlyLysPheGlyGlnSerGlySerGlySerSerAsnAsnAsnHisIleValGlyHisTyrThrHisThrTrpAlaValHisIleGlcProAsnGlyAsp 2460
AATGGCATGGCGGATCGGGTTGCCAAGGATCGGTCAATTTGGGCAAGATTCGTCAATTTGGGCAAGATTCGTATGATCATACCATTCACCAAGCTCGAAGGTCTGCTCTCCCCGCCACG 1080
AsnGlyMetAlaAspAlaValAlaLysAspHisGlyPheValAlaAsnLeuGlyLysIlePheAspAspHisTyrHisPheAlaHisHisLysValSerLysArgSerLeuSerProAlaThr 2880
CATCACAAACTCGCTGGATGACGACGATCGCTCCACTGGCGCAAGCAGCAGCGGCCCAAGTCGCGATCCAAACGGGACTTTATCCGCACTGCGACCTCAGGACCTCCTCGCAGGCC 1200
HisHisGlnThrArgLeuAspAspAspArgValHisTrpAlaLysGlnGlnArgAlaLysSerArgSerLysArgArgPheIleArgMetArgProSerArgThrSerSerArgAla 3200
ATGTCGATGGTGGACGCCATGCTCTTAACTGACTCCAAGTGGCCGAGATGTGGTATCTGAATCGTGGTGGTGGCTGGACATGAATGTGATACCCGCTGGAAGATGGGCATAACCGGC 1320
MetSerMetValAspAlaMetLysThrAsnAsnSerLysTrpProGlnMetTrpTyrLeuAsnArgGlyGlyGlyLysAspMetAsnValIleProAlaTrpLysMetGlyIleThrGly 3600
AAGGGCTGGTGGTGACAAATTCGGATGATGGCTGGAAATCCGATCCGAGCATACAGGATAACATACGATCCCAAAGCCTCGTAGCATGTGAATAGGCCACGACGACGATCCGATCCG 4000
LysGlyValValValThrIleLeuAspAspGlyGluLeuGluSerAspHisTrpGlyIleGlnAspAspTyrAspProLysSerTyrAspValAsnSerHisAspAspAspProMetPro 4400
CATTACGATATGACGGACTCGAACCGCCCATGGAATCTCGCTGTGCGCGGAGGTGGCAGCACCCGCGCAACAATTCGTTCTGCGCGGTGGGTATGGCTACGGCGCCAGTGTGGCGGAGT 1560
HisTyrAspMetThrAspSerAsnArgHisGlyThrArgCysAlaGlyGluValAlaAlaThrAlaAlaAsnSerPheCysAlaValGlyIleAlaTyrGlyAlaSerValGlyGlyVal 4400
AGGATCTGGACGGAGACGTACGGATCGGGTTGAGGCACGGTCGCTGTGCTGAATCCGCGACCATTCGACATATACAGTGCCTCCTGGGACCGCATGACGATGCGCAAGACGGTGGAG 1680
ArgMetAspGlyAspValThrAlaAlaValGluAlaArgSerLeuSerProGlnHisIleAspIleTyrSerAlaSerTrpGlyLysProAspAspGlyLysThrValAsp 4800
GGACCGCGGAACTGGCATCGCGGCCTTTATCGAGGCGCAACTAAGGGACCGCGCGCGCAAGGGCAGCATCTTCATATGGGCATCGGCGAATGGTGGCGGAGCAGGATAACTGCAAC 1800
GlyProGlyGluLeuAlaSerArgAlaPheIleGluGlyThrThryLysGlyArgGlyGlyGlySerIlePheIleTrpAlaSerGlyAsnGlyGlyArgGluGlnAspAsnCysAsn 5200
TGGCAGCGGTACACGAATCCATCTGGACGCTGTCCATCTCCAGTGCACGGAGGAGGCCATGTGCCCTGTACTCGGAGAAGTGCAGCTCCACGCTGGCCACCACTACAGCAGCGC 1920
CysAspGlyTyrThrAsnSerIleTrpThrLeuSerIleSerSerAlaThrGluGluGlyIleValProTrpTyrSerGluLysCysSerSerThrLeuAlaThrThrTyrSerSerGly 5600
GGCAGGGCGAGAGCAGGTGGTCACCGGACTGCACACTCGTGCACTGTCTCCACACGGGCACTCGGCGTGGCGCCCGCTGCGCCTGGCATAGCGGCCCTGGTGTCTGCAGTCC 2040
GlyGlnGlyGluLysGlnValValThrThrAspLeuHisHisSerCysThrValSerHisThrGlyThrSerAlaSerAlaProLeuAlaAlaGlyIleAlaAlaValLeuGlnSer 6000
AACCAGAATCTCACTTGGCGGATCTGCAGCAATTTGTCGCGCAGCCGCAAGCCGCGCAACTTAAGGACCCAGCTGGTCAGCAATGGGTGGGCGCGGAGTACGCCCTTT 2160
AsnGlnAsnLeuThrThrArgAspLeuGlnHisIleValValAlaThrThrAlaLysProAlaAsnLeuLysAspProSerTrpSerArgAsnGlyValGlyArgArgValSerHisSerPhe 6400
GGCTACGGATTATGGACGCCCGGAGATGTCGCGCTGGCCCGCACTGGAAGGCGGTGGCGAGCAGCAGCGTGCAGAGATTACGCTCCCCATGTGCACAAAGGTATTCCACCTCGT 2280
GlyTyrGlyLeuMetAspAlaAlaGluMetValArgValAlaArgAsnTrpLysAlaValProGluGlnGlnArgCysGluIleAsnAlaProHisValAspLysValIleProProArg 6800
ACCCATATCACTTCACTGACGGTTAATCACTGTGATCGGTCAATTACCTGGAGCAGCTCCAGGCCAAGATTACGTAACCTGCAGAGACGAGGAGACATTACGCTCTTTTGGAG 2400
ThrHisIleThrLeuGlnThrThrValAsnHisCysArgSerValAlaThrIleHisIleValGlnAlaLysIleThrLeuThrSerGlnArgArgLysIleGlnLeuAlaArg 7200
TCTCCCGCAAAACCACTGTCCAGCTCTCAACGCTAGGATACATGACAATCTCGTTCCGGATCAANTCAATGGCCCTTCATGCTGTGCACAGTGGGAGAGTCCGCCGCAAGGAAC 2520
SerProAlaAsnThrSerValThrLeuThrThrProArgIleHisAspAsnSerArgSerGlyPheAsnGlnTrpProPheMetSerValHisThrTrpGlyGluSerProGlnGlyAsn 7600
TGGCAGCTGGAGATCCACACAGAGGGTGCCTATATGGCAAAATCACAAATGGGATATGATATCTACGGCACGGAACGCCGCCCAACCCGATGACTGTGCCAATCCCGACGAGTCG 2640
TrpGlnLeuGluIleHisAsnGluGlyArgTyrMetAlaGlnIleThrGlnTrpAspMetIlePheTyrGlyThrGluThrProAlaGlnProAspAspValAlaAsnProSerGlnSer 8000
AACCAGTTCAATCTGACCGCAACGATATGGCCCACAATGACGTGCAGTACGATTCCACGGCCAGTGGAGGAATATGCAGCAGGTGGGCGAGTGGGCATGACCCGAGATCACAGCAAC 2760
AsnGlnPheAsnLeuTyrGlyAsnAspMetAlaHisAspAspValGluThrThrGlyGlnTrpArgAsnMetGlnGlnValGlyGluValGlyMetThrArgAspHisSerAsn 8400
ACCGCGCGCTGCTTAAGTGGAGCGATCGCAAGTGTAGAGTGCAATGACTCCGCTTATGTTCAGGACCACTGCTACAGTGTCTGCCGTGCACACATATCCATTTGGACAGTTC 2880
ThrAlaAlaCysLysLysTrpSerArgArgLysCysLeuGluCysAsnAspSerAlaTyrMetPheGluAspGlnCysTyrAspValCysProValHisThrTyrProLeuAspLysPhe 8800
CAAGCAGAGGAGGATGACGAGGATGACGAGGTGACAAGGGTCCGGTTAATCCCTACAGCAGCTCCCCATGGATCATTCCCTGTTGATGTCCAACGCTTGGATGACAAGCAAGATCT 3000
GlnAlaGluGluAspGluGlnAspAspGluValThrArgGlyProValAsnProTyrSerSerProMetAspHisSerLeuLeuMetSerAsnSerLeuAspAspLysGlnAspPro 9200
CTCAGCGCGGAGGATGACGCTGCTGTAGTTCTACTCACCACACTGGTGAGGTGCTTCTCGGGTCTGTGCGCCTGCGCATCGGAGCTGCTGGAGTGCTACGGTGCCTGCCCTCCGAC 3120
TgcGlnAlaGluAspArgArgArgSerSerThrGlnLeuValGluValProSerArgValCysAlaAlaCysAspArgSerLeuGluCysTyrGlyAlaSerGln 9600
TGCGACCTCTGCTCCCCGGGACGCAACTGCGTAAGATCTGAAAGCAGACCTTCTGCTATGCTGTGGTGCAGTACGGGAATGGCCGATGTGGTGGACATTTCCAAATGGACGAT 3240
CysSerAspThrSerProGlySerGlnLeuArgGlyIleLeuGlnGluThrPheCysTyrAlaTyrValArgSerThrGlyMetAlaSerValValAspIleSerLysMetAspAsp 10000
AGAGATACCCAGCAATATATGACTGGCACAATATGTGCTCTTTTGGTTTCGGTGATTTTCACTGATGGGCGTAGCAGTGGCCGCTGGCATCGTGTATCATCGAAGGCAATGGCTCGA 3360
ArgAspThrGlnGlnTyrMetThrGlyThrIleValLeuLeuValSerValIlePheThrLeuMetGlyValAlaValAlaGlyGlyIleValTyrHisArgArgAlaMetAlaArg 10400
TCCACGAACTATCTCCGAGTTTCCCTAGTCCCGGTGACGAAAGTGACTCTGACGAGGATGAACGTGTTACGGCCCACTCCACGCGAGAAAGATGGTGTAAATATATATCCGCGAT 3480
SerAsnGluLeuTyrSerArgValSerLeuValProGlyAspGluSerAspSerAspGluAspGluLeuPheThrAlaHisPheProAlaArgLysSerGlyValAsnIleTyrArgAsp 10800
GAAGCGCCGAGGAAAGATATTCGAGGAAGATGAGTACGCACTTGGTACCTTAATAATCAAAACAGTTATTAGTTAGGTCATGCATTAGGCATCCTCATAACCCAGCAAAAT 3600
GluAlaProSerGlnLysIlePheGluGlnAspGluIleThrThrSerHisLeuValPro ***** 1100
ATCTATATGTTAAGCAACCGCAACACGCAATTTTGGTCTATATTTTGTACATTGACGGCATACAGCTCCTTTATTCAAGCGAAAATAGATTAAAGATTAAATGTAAGCAAAAAA 3720
AATCAAACTAATTTTGTAAATTTTATGCTTAAATAAATGATTTTAACTGAAAAA *****
***** 3740

3781

sites of autoproteolytic maturation, a property of subtilisin proteases (Power et al., 1986; Ikemura and Inouye, 1988). Another feature of the predicted dKLIP-1 protein that is conserved with PC2, PC3, and furin is an Arg-Gly-Asp (RGD) motif at amino acid residues 715–717. The RGD sequence, initially identified in fibronectin, is required for integrin binding and cellular adhesion (Pierschbacher and Ruoslahti, 1984).

dKLIP-1 is translocated across membranes

To determine whether the dKLIP-1 polypeptide, which appeared to lack an amino-terminal signal sequence, could be translocated across microsomal membranes, a series of *in vitro* translation and translocation experiments were performed. Capped dKLIP-1 RNA was synthesized *in vitro* and translated in reticulocyte lysate reactions. SDS-PAGE analysis of the translation products showed a predominant protein product of approximately 140 kDa (data not shown), in good agreement with the predicted size (131 kDa) of the dKLIP-1 protein. To test whether the dKLIP-1 translation product was translocated across microsomal membranes, *in vitro* translation/translocation reactions were performed in the presence of membranes followed by protease treatment with or without detergent. As occurred in the absence of membranes, translation of the dKLIP-1 RNA in the presence of microsomal membranes also resulted in the synthesis of a 140 kDa protein (Fig. 4, lane 1). Treatment of a replicate sample with proteinase K resulted in a 130 kDa translation product that was protected from protease digestion (Fig. 4, lane 2). In contrast, when the translation/translocation reactions were protease treated in the presence of detergent to disrupt microsomal membranes, the protein was completely degraded (Fig. 4, lane 3). These results demonstrate that the dKLIP-1 cDNA encodes a 140 kDa protein that can be translocated across microsomal membranes.

dKLIP-1 is proteolytically active in vivo

Previously both Kex2p and hFurin were shown to enhance greatly the endoproteolytic conversion of murine pro- β -NGF to mature 13 kDa β -NGF *in vivo* (Bresnahan et al., 1990). To assess the proteolytic activity of dKLIP-1, the protein was coexpressed with pro- β -NGF in cultured cells using VV vectors, and the conversion of the precursor to mature β -NGF was analyzed.

Replicate wells of BSC-40 cells were co-infected with a VV recombinant expressing murine pro- β -NGF (VV:mNGF) and either wild-type VV (VV:WT) or a vaccinia recombinant expressing dKLIP-1 (VV:dKLIP-1). Pulse-labeled products secreted into the medium were immunoprecipitated with an anti- β -NGF polyclonal antiserum and fractionated by SDS-PAGE. The results show that predominantly 40 kDa pro- β -NGF was secreted from cells co-infected with VV:mNGF and VV:WT (Fig. 5B, lane 1). However, when cells were co-infected with VV:mNGF and VV:dKLIP-1, pro- β -NGF was completely con-

Table 1. Percentage overall amino acid sequence homology

Kex2p homolog	Percentage dKLIP-1
hFurin	48/65
mPC3	46/63
hPC2	44/62
Kex2p	29/52
Subtilisin	26/46

Data are presented as amino acid identities/similarities, the latter defined by evolutionary distance between the amino acids (Gribskov and Burgess, 1986).

verted to mature 13 kDa β -NGF (Fig. 5B, lane 2). To determine the site of cleavage within the precursor, the 13 kDa peptide generated from coexpression of dKLIP-1 and pro- β -NGF (Fig. 5B, lane 2) was transferred to a PVDF membrane and sequenced by Edman degradation (Matsudaira, 1987). Release of 35 S radioactivity was detected in the ninth cycle, which is consistent with the position of the first methionine within mature β -NGF formed by cleavage of the precursor at Lys¹¹⁹–Arg¹²⁰ (Fig. 5C). Thus, dKLIP-1 greatly enhanced conversion of the precursor to β -NGF peptide, by cleavage at a pair of basic amino acids.

Expression of dKLIP-1 transcripts in adult tissues

To determine the size of the dKLIP-1 hybridizing transcript(s), Northern blots of polyA⁺ RNA isolated from adult *Drosophila* heads and bodies were hybridized to a dKLIP-1 cDNA probe. Transcripts of 4.0, 4.5, and 6.8 kb were present in both heads and bodies at similar levels (Fig. 6A). However, an 8.4 kb transcript was preferentially expressed in heads.

Determination of the anatomical distribution of dKLIP-1 transcripts in adult tissues was performed on 8 μ m horizontal sections of male and female flies hybridized to 35 S-labeled riboprobes. Antisense probes bound specifically to a number of tissues (Fig. 7A,D,F), while control sense-strand probes showed low, nonspecific signals (Fig. 7C). In the head (Fig. 7A,B), high levels of hybridization were present in all cortical regions of the brain containing neuronal cell bodies. In contrast, the neuropil, which contains cellular processes, showed only background levels of hybridization. A similar pattern was observed in neuronal cell bodies in the ventral ganglion (data not shown). Lower but significant levels of hybridization were present in the fat body (Fig. 7A,B).

Strikingly, maternally synthesized dKLIP-1 mRNA was detected in nurse cells and oocytes (Fig. 7D–F). In addition, follicle cells showed low levels of hybridization. The highest overall signals were observed in the oviduct (Fig. 7D–F). These results demonstrate that dKLIP-1 mRNAs are expressed in the adult CNS, fat body, and female reproductive tissues.

←

Figure 2. Nucleotide sequence of the dKLIP-1 cDNA and deduced protein sequence. *A*, Schematic diagram showing two overlapping cDNA clones, 1L and 3, isolated from an adult *Drosophila* head-specific cDNA library. The approximate length of sequence determined from subcloned fragments is represented by arrows. The lower diagram of the panel shows the ORF (thick line) containing the subtilisin-like catalytic domain (shaded box) in relation to the composite cDNA. The composite dKLIP-1 cDNA was generated by ligating 784 bp from clone 1L to 2997 bp from clone 3 using the BamHI (*B*) restriction site. *B*, The nucleotide sequence of the dKLIP-1 cDNA. An opa repetitive element (nt 257–347) (Wharton et al., 1985) is indicated by single thin underline between the nucleotide and amino acid sequences. Ten potential N-linked glycosylation sites (Asn–X–Ser/Thr) are indicated by thick underlines. Putative membrane spanning domains are boxed. Open triangles, potential paired basic residue autoproteolytic maturation sites; asterisks, His, Asp, and Ser residues that form the catalytic triad, as well as the Asp residue that stabilizes the transition state complex with the substrate (Kraut, 1977); double underline, RGD sequence important for cellular adhesion; dots, AUUUA motif important for conferring RNA instability. The polyadenylation addition site (nt 3755–3760) is underlined, also.

Figure 3. Structural features and sequence alignment analyses of the dKLIP-1 polypeptide with Kex2p-like homologs. *A*, Kyte and Doolittle hydropathy plot as performed by the PEPLOT program (Devereux et al., 1984). The two hydrophobic regions that represent potential membrane spanning domains (residues 118–151 and 1006–1036) are hatched. *B*, Schematic diagram showing structural features and relative lengths of dKLIP-1, hfurin, yeast Kex2p, hPC2, and mouse PC3 polypeptides. Ovals, potential N-linked glycosylation sites. Signal sequences are marked with striped boxes. Putative TMDs are marked by hatched boxes. Potential paired basic amino acid autoproteolytic cleavage sites (see Discussion) are marked

Figure 4. *In vitro* translocation of dKLIP-1 across microsomal membranes. Replicate tubes containing capped dKLIP-1 transcripts, rabbit reticulocyte lysate, and ^3H -leucine were incubated for 30 min at 30°C. Samples were then incubated in the absence (lane 1) or presence of 0.1 mg/ml proteinase K alone (lane 2) or with 0.1% Triton X-100 (lane 3) for 45 min on ice. SDS-PAGE analysis of the products and molecular weight standards was performed as described in Materials and Methods.

The expression of dKLIP-1 transcripts is developmentally regulated

Based on the observation that dKLIP-1 mRNA was detected in developing oocytes, expression of the gene during *Drosophila* development was assessed by Northern blot analysis. PolyA⁺ RNA from embryonic, larval, and pupal stages was blotted and hybridized to a dKLIP-1 cDNA probe (Fig. 8*A*). A 4.0 kb RNA transcript was present at the earliest embryonic stages [0–2 hr postfertilization (pf)] (Fig. 8*A*, lane 1). From 2–10 hr pf, the levels of all dKLIP-1 transcripts were below detection. At 10–14 hr pf, 4.5, 6.8, and 8.4 kb transcripts were evident, with the 4.5 kb form being most abundant (Fig. 8*A*, lane 4). From 14 hr pf through pupal day 4, all dKLIP-1 transcript levels were again below detection.

The spatial localization of dKLIP-1 transcripts during embryogenesis was assessed by *in situ* hybridization of ^{35}S - and digoxigenin-labeled dKLIP-1 probes to embryonic tissue sections and whole-mounts, respectively. Hybridization was uniformly distributed in stages 1–4 (0–2.5 hr pf) embryos (Fig. 9*A,B* and data not shown). The same photomicrograph shows a stage 10 embryo (5–7 hr pf) that has undergone germ-band extension. Significantly reduced levels of hybridization were present at this stage as expected from Northern blot analysis (described above). This reduction in signal during embryogenesis was observed by stage 5 (cellular blastoderm formation, 2.5–3.25 hr pf) (data not shown). Elevated levels of hybridization reappeared at stage 14 (11.5–13 hr pf), when signals were found in the anal pads, the

hindgut, brain and ventral ganglia (Fig. 9*C–F*). Expression in the anal pads and hindgut was transient, disappearing by stage 16 (13–16 hr pf). In contrast, expression in the CNS continued through stage 16, when it was found in whole-mount preparations to be in the ventral ganglia in 12 sets of cell clusters located along the ventral midline and ventrolaterally (Fig. 10).

Discussion

The predicted dKLIP-1 polypeptide has unique structural features

The nucleotide sequence of the dKLIP-1 cDNA (Fig. 2) predicted an 1101 amino acid polypeptide containing the conserved Asp, His, and Ser catalytic triad residues characteristic of the subtilisin family of serine endoproteases. dKLIP-1 is structurally similar to a recently identified group of mammalian Kex2-like precursor processing endoproteases, furin, PC2, and PC3. Comparison of the predicted amino acid sequence of dKLIP-1 with the other Kex2p-like endoproteases revealed a high degree of sequence identity, particularly within the subtilisin-like catalytic domain (Fig. 3*B,C*; Table 1). In addition, alignment of the catalytic domains resulted in a similar positioning of the C-terminal transmembrane domains in dKLIP-1, hFurin, and Kex2p as well as shared positions for a small cluster of paired basic amino acids. In the case of Kex2p, one of these cleavage sites represents a proteolytic maturation site (Fuller et al., 1991), which is conserved at nearly the identical position in each of the other endoproteases (Fig. 3*B*).

There are, however, some marked differences between the

← as solid vertical bars. The subtilisin-like catalytic domains, with the catalytic triad residues (D, Asp; H, His; S, Ser), are shown by the shaded boxes. C, Amino acid alignment and consensus sequence of yeast Kex2p (*yKex2*), dKLIP-1, hFurin, and hPC2 catalytic domains using the BESTFIT program (Devereux et al., 1984). *, Residues important for substrate catalysis. The Asn residue involved in transition state hydrogen bonding is conserved by dKLIP-1 but changed to an Asp residue in PC2 (Smeekens and Steiner, 1990).

Figure 5. *In vivo* proteolytic processing of pro- β -NGF by dKLIP-1. **A**, Schematic diagram showing the precursor structure for the 40 kDa pro- β -NGF (above) (Darling et al., 1983) as expressed from the VV:mNGF vector and its conversion to 13 kDa β -NGF (below). The signal sequence is hatched and potential paired basic amino acid cleavage sites are shown as solid vertical lines. **B**, SDS-PAGE and fluorogram of immunoprecipitated products from pulse-labeled BSC-40 cells co-infected with VV:mNGF plus VV:WT (lane 1) or VV:mNGF plus VV:dKLIP-1 (lane 2). After a 30 min methionine depletion, cells were metabolically labeled with 35 S-methionine for 3 hr. Equal volumes of media samples were then incubated with a β -NGF antiserum (Lara et al., 1990), and the immunoprecipitated products were analyzed as described in Materials and Methods. The glycosylated precursor migrates as a broad band of ~35–40 kDa, while mature β -NGF migrates at 13 kDa. Products at 14, 16, and 17 kDa are probably processing intermediates. **C**, Sequential Edman degradation analysis of β -NGF from pulse-labeled BSC-40 cells co-infected with VV:mNGF and VV:dKLIP-1. Cells were labeled for 3 hr with 35 S-methionine, and immunoprecipitated products were separated by SDS-PAGE. Following transfer to a PVDF membrane (Immobilon-p, Millipore) and autoradiography, the 13 kDa band was subjected to sequential rounds of Edman degradation, from which aliquots were counted by liquid scintillation. The predicted N-terminal sequence of β -NGF is shown below each sequential cycle.

Figure 6. Northern blot analysis of head and body RNA from adult flies. **A**, Polyadenylated RNA (10 μ g/lane) from heads and bodies of both sexes was isolated separately, blotted, and hybridized with a 32 P-labeled dKLIP-1 cDNA probe as described in Materials and Methods. Size markers are labeled in kilobases. **B**, The same blot stripped and rehybridized with a 32 P-labeled cDNA probe for the ribosomal protein RP49 to quantitate relative RNA load per lane.

structure of dKLIP-1 and the other Kex2p homologs. First, each of the previously reported homologs is similar in size. In contrast, the translation product encoded by dKLIP-1 is 287 residues longer than Kex2p. Alignment of the catalytic domain of dKLIP-1 with the catalytic domains of the other Kex2p-like endoproteases suggests that the larger size of dKLIP-1 is apparently due to an extended amino-terminal sequence (Fig. 3B). Correspondingly, this amino-terminal extended domain of dKLIP-1 contains the least amount of sequence conservation compared with the other Kex2p homologs. Second, whereas each of the other Kex2p-like endoproteases possesses an N-ter-

Figure 7. *In situ* hybridization to adult head and ovary sections. Anterior is up in A–C, to the left in D–F. A and B, Dark-field and bright-field images, respectively, showing localization of dKLIP-1 mRNA using an antisense probe in a female head with high levels of hybridization in cortical brain regions (arrows), and the fat body (F). The central neuropil (NP) of the brain has a background level of signal (compare with C). Low levels of hybridization in the eyes (E) are slightly above background and therefore are not a reliable indication of dKLIP-1 message. The high levels of signal outlining the head and abdomen result from nonspecific binding of the probe to the extracellular cuticle and are a common artifact of the preparation. C, Dark-field image of an adjacent head section hybridized with a sense-strand dKLIP-1 probe as a control shows low or background levels of hybridization. D and E, Dark-field and bright-field images of a female abdomen hybridized with antisense probe showing localization of dKLIP-1 message to the ovaries including nurse cells (N), oocytes (O), and the oviduct (OV). F, High-magnification image of the region surrounding the oviduct (OV), where the highest levels of expression are detected. Scale bars, 200 μ m.

minimal signal sequence to permit transit into the secretory pathway, dKLIP-1 lacks this domain. Nonetheless, when translated *in vitro*, dKLIP-1 was translocated across microsomal membranes (Fig. 4). In addition, coexpression of dKLIP-1 *in vivo* resulted in the complete conversion of pro- β -NGF at –Lys¹¹⁹–Arg¹²⁰– in the secretory pathway (Fig. 5B,C). Possibly, the internal hydrophobic domain (residues 118–151) unique to dKLIP-1 facilitates its translocation into the lumen of the endoplasmic reticulum.

dKLIP-1 expression is developmentally regulated

In situ studies of adult fly sections detected dKLIP-1 transcripts in ventral ganglia and brain as well as the fat body (Fig. 7A,B). However, most striking was the detection of dKLIP-1 transcripts in ovaries (Fig. 7D–F). Expression in developing oocytes and nurse cells demonstrated that these dKLIP-1 transcripts are of maternal origin and implies a role for dKLIP-1 in early embryogenesis, prior to zygotic transcription.

Figure 8. Temporal expression of dKLIP-1 transcripts during *Drosophila* development. **A**, Polyadenylated RNA (10 μ g/lane) isolated from indicated developmental stages grown at 25°C was blotted and hybridized with 32 P-labeled dKLIP-1 cDNA probe. The adult RNA (lane 11) was a mixture of the RNA analyzed in the adult head/body blot (Fig. 6). Size standards are indicated in kilobases to the left. **B**, Control hybridization using RP49 as an internal standard for quantitative RNA load per lane.

Detection of dKLIP-1 RNA in 0–2 hr embryos (Figs. 8*A*, lane 1; 9*A*, *B*) is associated with the expression of a variety of transcripts and proteins involved in the determination of polarity and positional information in the embryo (Ingham, 1988). Later in development, dKLIP-1 transcripts are expressed in embryos at discrete times and restricted to specific cell populations. After germ-band shortening (10–14 hr), RNA levels were detected in the hindgut, ventral ganglia, brain, and anal pads (Figs. 9*C–F*, 10). During this time, the hindgut lengthens and the neuropil forms in the nervous system.

After stage 4, dKLIP-1 transcripts appeared to be rapidly degraded (Fig. 8*A*, lanes 1, 2). The loss of signal suggests that dKLIP-1 expression is developmentally regulated, in part, by rapid turnover of its RNA. dKLIP-1 and several mammalian mRNAs (e.g., GM-CSF, c-fos, and c-myc) possess a motif (AUUUA) repeated within their 3' UTRs (Shaw and Kamen, 1986; Fig. 2). Mammalian transcripts possessing this motif are selectively and rapidly degraded.

In addition to the AUUUA sequence in the 3' UTR, the dKLIP-1 transcript contains an opa element (Fig. 2, nt 258–347), a repeated trinucleotide (CAX_n) located in the nonconserved amino-terminal domain of the protein coding sequence (Wharton et al., 1985). Although the function of this element is unknown, opa sequences are present in several developmentally regulated *Drosophila* genes including *notch*, *antennapedia*, and *dorsal* (Grabowski et al., 1991).

Roles for serine proteases in early fly development and potential substrates for dKLIP-1

Numerous studies have demonstrated that trypsin-like activities and other proteins with homology to mammalian serine proteases have essential roles in fly development. During larval

metamorphosis, for example, imaginal disk eversion appears to be regulated by a trypsin-like protease activity since trypsin treatment of disks in culture increased the rate of eversion (Pino-Heiss and Schubiger, 1989). Conversely, trypsin inhibitors retarded this process. During early embryogenesis, dorsal-ventral axis formation is mediated by a biochemical pathway that includes the products of two genetic loci, *easter* and *snake* (DeLotto and Spierer, 1986; Chasan and Anderson, 1989). The products of both loci are structurally related to mammalian trypsin/chymotrypsin serine proteases and include the His, Asp, and Ser residues forming the characteristic catalytic triad (as opposed to the Asp, His, and Ser order in the subtilisin-like triad) (Neurath, 1984).

Unlike the trypsin/chymotrypsin family of endoproteases, the Kex2p-like subtilisin endoproteases specifically cleave substrates on the C-terminal side of paired basic amino acids (–Lys–Arg–, –Arg–Arg–) in the secretory pathway (Thomas et al., 1988a, 1991; Fuller et al., 1989a; Mizuno et al., 1989). Analysis of mutant substrates (Bentley et al., 1986; Yoshimasa et al., 1988, 1990; Yoshiro and Webster, 1988) suggests that an additional feature of the cleavage site commonly found in many precursor proteins is an arginine located four residues (–4 Arg) N-terminal to the paired basic cleavage site (–Arg–X–Lys/Arg–Arg–). Indeed, studies *in vitro* indicate a strong preference by dKLIP-1 for synthetic peptide substrates containing a pair of basic residues and a –4 Arg (J. S. Hayflick and G. Thomas, unpublished observations).

Several developmentally important *Drosophila* precursor proteins expressed coincident with dKLIP-1 during embryogenesis possess the –Arg–X–Lys/Arg–Arg– cleavage site motif and are thus candidate substrates for a Kex2p-like endoprotease such as dKLIP-1. These include the gene products of the *decapen-*

Figure 9. *In situ* localization of dKLIP-1 transcripts in 8 μ m sections of embryos. *A* and *B*, Dark-field and bright-field images of an early nuclear cleavage (stage 1; ventral, right; anterior, down) embryo and a germ-band extended (stage 10; ventral, left; anterior, up) embryo. Note the elevated signal in the stage 1 embryo compared to the background levels seen in the stage 10 embryo. *C* and *D*, Dark-field and bright-field images of a horizontal section through a stage 14 embryo (anterior, right) showing signals located in the anal pads (arrows). *E* and *F*, Dark-field and bright-field images of a parasagittal section through a stage 14 embryo (ventral, down; anterior, right) showing signals in the brain (*B*), hindgut (*H*), ventral ganglia (*V*), and anal pads (arrows). The central bright material in *C* and *E* is yolk (*Y*) and is an artifact of the preparation. Scale bars, 100 μ m.

Figure 10. *In situ* hybridization detection in a stage 16 whole-mount embryo using an antisense digoxigenin-labeled probe. The signals are localized to three rows of cell bodies, two lateral (arrowheads) and one medial, in the ventral ganglia (anterior, left). Scale bar, 100 μ m.

taplegic and *tolloid* loci as well as an insulin-like pro-receptor protein (Nishida et al., 1986; Padgett et al., 1987; Shimell et al., 1991).

Biochemical studies based on expression of *decapentaplegic* translation product (dpp) in Schneider 2 (S2) cells resulted in the cleavage of pro-dpp on the COOH-terminal side of a single basic residue with a -4 Arg (Panganiban et al., 1990). A conserved paired basic residue site with a -4 Arg (-Arg-Asn-Lys-Arg-), proximal to the cleavage site in pro-dpp was not utilized as it is in the structurally related pro-TGF β 1 and the inhibin precursors (Derynck et al., 1985; Mason et al., 1985). It has not been reported if processing of pro-DPP in S2 cells mimics the endogenous processing in the developing embryo. Studies examining the ability of dKLIP-1 to process these precursors are currently underway.

The cloning and characterization of the *Drosophila* Kex2p homolog dKLIP-1 now afford the exploitation of *Drosophila* genetics to elucidate the role of this endoprotease and its metazoan homologs *in vivo*.

Note added in proof. The DNA sequence reported in this paper has been deposited in the Genbank database (accession number M81431). The dKLIP-1 gene localization and related molecular cloning information have been deposited in the *Drosophila* Genmaps database under KexZ-like endoprotease-1.

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