Loss of Secretory Pathway FK506-binding Proteins Results in Cold-sensitive Lethality and Associate Extracellular Matrix Defects in the Nematode Caenorhabditis elegans*

Alan D. Winter†, Sylvain C. P. Eschenlauer‡, Gillian McCormack§, and Antony P. Page‡†

From the †Institute of Comparative Medicine, Faculty of Veterinary Medicine, The University of Glasgow, Bearsden Road, Glasgow G61 1QH, Scotland, United Kingdom and the §Wellcome Centre for Molecular Parasitology, The University of Glasgow, Glasgow G12 8TA, Scotland, United Kingdom

The FK506-binding proteins (FKBs) represent ubiquitous enzymes that catalyze the rate-limiting peptidyl prollyl cis-trans isomerization step in protein folding. The nematode Caenorhabditis elegans has eight FKBs, three of which (FKB-3, -4, and -5) have dual peptidyl prollyl cis-trans isomerase (PPIase) domains, signal peptides and ER retention signals. PPIase activity has been detected for recombinant FKB-3. Both FKB-3 and -5 are expressed in the exoskeleton-synthesizing hypodermis with transcript peaks that correspond to the molting and collagen synthesis cycles. FKB-4 is expressed at a low level throughout development. No phenotypes were observed in deletion mutants in each of the secretory pathway FKBs. Combined triple and fkb-4, -5 double deletion mutants were however found to arrest at 12 °C, but developed normally at 15–25 °C. This cold-sensitive larval lethal effect was not maternally derived, occurring during embryogenesis, and could be rescued following the transgenic introduction of a wild type copy of either fkb-4 or fkb-5. The temperature-sensitive defects also affected molting, cuticle collagen expression, hypodermal seam cell morphology, and the structural integrity of the cuticular extracellular matrix. This study establishes that the secretory pathway FK506-binding PPIase enzymes are essential for normal nematode development, collagen biogenesis, and the formation of an intact exoskeleton under adverse physiological conditions.

1 To whom correspondence should be addressed: Tel.: 0044-141-3301997; Fax: 0044-141-3305603; E-mail: a.page@vet.gla.ac.uk.

* This work was supported by the Medical Research Council UK through Senior Fellowship Award G117/476 (to A. P. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: FKB or FKBP, FK506-binding protein; ECM, extracellular matrix; WT, wild type; PPIase, peptidyl prollyl cis-trans isomerase; CYP, cyclophilin; DIC, differential interference contrast; ER, endoplasmic reticulum.

‡ The FK506-binding proteins (FKBPs or FKBs) belong to a group of proteins that have peptidyl prollyl cis-trans isomerase (PPIase, EC 5.2.1.8) activity, and together with the cyclophilins (CYPs or CYNs) are collectively called the immunophilins. FKBs and CYPs are structurally unrelated and have high affinities for the structurally distinct immunosuppressive drugs FK506 and cyclosporin A, respectively (1). In addition, there is no link between PPIase activity and the immunosuppressive action of these compounds. The immunophilins have a widespread distribution in nature being found in bacteria, plants, and man. However, the endogenous physiological functions are poorly understood but include possible roles in protein translation, folding, assembly, and trafficking (1, 2). These enzymes stabilize the cis-trans transition state, accelerate the isomerization event and therefore promote protein folding and assembly of multiprotein complexes.

The collective roles of the FKBs and CYPs have been addressed following the generation of single and multiple mutants in the budding yeast Saccharomyces cerevisiae. No overt phenotypes were observed, indicating that they are dispensable for normal development in this simple unicellular organism (3). The examination of immunophilin function in multicellular organisms has likewise been relatively uninformative raising the possibility of redundancy of function and the possible non-essential nature of these genes. In relation to PPIase activity, this is supported by the fact that proline isomerization will proceed in the absence of a PPIase catalytic, albeit at a slow rate, particularly at lower temperatures (4, 5). The major exception to the lack of associated phenotype for an immunophilin mutant has been described for the ninaA cyclophilin gene of Drosophila. This gene product is involved in rhodopsin folding, and mutant forms result in protein misfolding leading to blindness (6, 7).

cis-trans Isomerization of peptidyl prollyl bonds has been established as being a slow rate-limiting step in the folding of numerous proteins, in the particular proline-rich collagens (8, 9) and tropoelastin (10). Nematode worms are encased in a collagen-rich extracellular matrix (ECM) called the cuticle that is synthesized in its entirety five times during normal development. The free living nematode Caenorhabditis elegans has been proposed as an excellent model system in which to dissect ECM formation (11) and to study the enzymes and chaperones involved in its biogenesis and deposition (12). Collagens represent essential structural proteins in all multicellular animals and are characterized by the repeat motif Gly-X-Y, where X and Y are commonly proline and hydroxyproline, respectively. The cuticle collagens represent a large multigene family that encodes small proline-rich collagens with interrupted triple helical regions. The collagens are synthesized in waves of expression that correspond to the molting cycle, a process that permits growth and defines the separate larval and adult forms and their distinct cuticles. The completely sequenced C. elegans
genome encodes eight separate FKB genes, five of which are involved in the secretory pathway (1). Of these isoforms, FKB-3, -4, and -5 have dual PPIase domains, a signal peptide, and an ER retention signal sequence. Here we examine the individual and collective functional significance of these FKBs in *C. elegans*. The collective phenotypes together with the shared temporal and spatial expression patterns confirm that an essential role is played by these enzymes in the folding of the nematode collagen and the biogenesis of the ECM.

**EXPERIMENTAL PROCEDURES**

**Nematode Strains and Generation of Deletion Mutants**—The *fkb-3(tm348)* homozygous viable deletion allele was isolated by Dr. Shohei Mitani, National Bioresource Project for the Nematode, using UV/TMP as a mutagen. A 1487-base pair deletion in C05C8.3 (*fkb-3*) on chromosome V, from -1128 to +360 relative to ATG start of *fkb-3* gene was created, that removed the promoter region plus all of the first exon of *fkb-3*. This is predicted to be a null mutant. The strain TP8 was made by outcrossing five times with wild type N2 nematodes using the following primers: tm348F1, 5′-gagcaatcgatagctgcagtgtaa-3′; tm348F2, 5′-ctctggaagaaatgcagat-3′; tm348R1, 5′-ctctggaaggctgcagat-3′. The *fkb-3(tm348)* homozygous viable deletion mutant in ZC455.10 on chromosome V was isolated using the mutagen UV/TMP following published protocols (13). (Selection screen primers are available on request.) The deletion region was cloned and sequenced revealing a 527-bp deletion in C05C8.3 (*fkb-3*). A 446-bp deletion on chromosome X, from 5417 to 5957 relative to the ATG, was obtained from the International *C. elegans* Gene Knock-out Consortium. The deletion site was cloned and sequenced using primers ILS 5′-tcggagaaagagacagcacc-3′ and ok240F1 5′-caggaatgatcgcgtaattctc-3′ and found to extend from -1478 to +48 relative to the ATG start of *fkb-4*. The strain RB1213 was then backcrossed four times to the N2 strain using the following primers: ok240F1, ok240R1, 5′-catagcttctctatcctaatggg-3′; ok240R2, 5′-gcggctgagttatagccacctg-3′. A *fkb-5(tm475)* homozygous viable deletion allele was isolated and obtained from Dr. Shohei Mitani. The allele *tm475* is a 446-bp deletion on chromosome I covering C05F2.6 (*fkb-5*) removing 237 bp of the *fkb-5* sequence, starting in the last intron and completely removing all 180 bp of the fourth exon and 209 bp of the downstream sequence.

**Reporter Gene Fusion: Spatial Analysis**—The *fkb-3, -4,* and -5 reporter gene plasmids were constructed using the *C. elegans* lacZ promoterless reporter gene expression vector pPD95.03 or pPD96.04 (Addgene). Reporter gene constructs comprised 1.5–2 kb of the potential upstream regulatory region and part of the first exon (or part of the first and second exons in the case of *fkb-4* construct 2) fused in a translationally in-frame context to the *lacZ* reporter gene. Constructs were generated from genomic DNA template by PCR with the following primers: *fkb-3* construct (from -1966 to +23 relative to the ATG), fkb3pF(HindIII) 5′-gacgcaactttgagttttctcaccgaggctttg-3′ and fkb-3pR (BamHI) 5′-gaggaattcgcagttttgtgttgttttgg-3′; *fkb-4* construct 1 (from -247 to +772 relative to ATG, removing the majority of exons 2 and 3). The strain TP65 was generated following backcrossing four times to N2 using the following primers: Fkb-4BCF1, cgaaccacttgtttcctg-3′; Fkb-4BCF2, 5′-gatcagatttcacaagattgaag-3′; Fkb-4BCR1, 5′-cattatcatatgctcctgg-3′. An additional homozygous viable deletion mutant, named *fkb-4(ok240)*, was obtained from the International *C. elegans* Gene Knock-out Consortium. The deletion site was cloned and sequenced using primers ILS 5′-tcgaagaaagacagcacc-3′ and ok240F1 5′-caggaatgatcgcgtaattctc-3′ and found to extend from -1478 to +48 relative to the ATG start of *fkb-4*. The strain RB1213 was then backcrossed four times to the N2 strain using the following primers: ok240F1, ok240R1, 5′-catagcttctctatcctaatggg-3′; ok240R2, 5′-gcggctgagttatagccacctg-3′. A *fkb-5(tm475)* homozygous viable deletion allele was isolated and obtained from Dr. Shohei Mitani. The allele *tm475* is a 446-bp deletion on chromosome I covering C05F2.6 (*fkb-5*) removing 237 bp of the *fkb-5* sequence, starting in the last intron and completely removing all 180 bp of the fourth exon and 209 bp of the downstream sequence.

**Construction of *C. elegans* fkb-3, fkb-4, and fkb-5 Promoter: Reporter Gene Fusion: Spatial Analysis**—The *fkb-3, -4,* and -5 reporter gene plasmids were constructed using the *C. elegans* lacZ promoterless reporter gene expression vector pPD95.03 or pPD96.04 (Addgene). Reporter gene constructs comprised 1.5–2 kb of the potential upstream regulatory region and part of the first exon (or part of the first and second exons in the case of *fkb-4* construct 2) fused in a translationally in-frame context to the *lacZ* reporter gene. Constructs were generated from genomic DNA template by PCR with the following primers: *fkb-3* construct (from -1966 to +23 relative to the ATG), fkb3pF(HindIII) 5′-gaggaattcgcagttttgtgttgttttgg-3′ and fkb-3pR (BamHI) 5′-gaggaattcgcagttttgtgttgttttgg-3′; *fkb-4* construct 1 (from -247 to +772 relative to ATG, removing the majority of exons 2 and 3). The strain TP65 was generated following backcrossing four times to N2 using the following primers: Fkb-4BCF1, cgaaccacttgtttcctg-3′; Fkb-4BCF2, 5′-gatcagatttcacaagattgaag-3′; Fkb-4BCR1, 5′-cattatcatatgctcctgg-3′. An additional homozygous viable deletion mutant, named *fkb-4(ok240)*, was obtained from the International *C. elegans* Gene Knock-out Consortium. The deletion site was cloned and sequenced using primers ILS 5′-tcgaagaaagacagcacc-3′ and ok240F1 5′-caggaatgatcgcgtaattctc-3′ and found to extend from -1478 to +48 relative to the ATG start of *fkb-4*. The strain RB1213 was then backcrossed four times to the N2 strain using the following primers: ok240F1, ok240R1, 5′-catagcttctctatcctaatggg-3′; ok240R2, 5′-gcggctgagttatagccacctg-3′. A *fkb-5(tm475)* homozygous viable deletion allele was isolated and obtained from Dr. Shohei Mitani. The allele *tm475* is a 446-bp deletion on chromosome I covering C05F2.6 (*fkb-5*) removing 237 bp of the *fkb-5* sequence, starting in the last intron and completely removing all 180 bp of the fourth exon and 209 bp of the downstream sequence.

**Maintenance and Manipulation of Nematodes**—Nematode strains were maintained following standard culture methods (14). L1 cultures were synchronized following bleach treatment of gravid hermaphrodites and single worm PCR was performed as described previously (13). The combined mutant strains
Transgenic Rescue of Cold-sensitive Lethal Mutants—Rescue of TP86 (fkb-4, fkb-5) and TP83 (fkb-3, fkb-4, fkb-5) deletion mutants with transgenic fkb-4 or fkb-5 was performed by microinjection. The fkb-4 rescue clone was generated by PCR on N2 genomic DNA with primers Fkb-4 NPF and Fkb-4 Rsp. The PCR products were cloned into pMalc2 vector (New England Biolabs). The primers used were as follows: fkb-3, fkb3rtF 5′-taacagctgccgtcatggcctag-3′ and fkb3rtR 5′-gacctgtggtgtaaaccac-3′; fkb-4, fkb4rtF 5′-taagacggaggtgaaatg-3′ and fkb4rtR 5′-tcctcttcacatcctc-3′; fkb-5, fkb5rtF 5′-gccgccgagagaccatg-3′ and fkb5rtR 5′-tagaaatggtggaataac-3′. The PCR reactions were electrophoresed, Southern-blotted, and probed with labeled DNA using co-injected Fkb marker, maintained at the permissive temperature of 20 °C for 5–10 transgenic animals were shifted to 12 °C. Controls included the original strains TP86 and TP83 were also shifted and assessed microscopically.

Temporal Analysis by Semiquantitative RT-PCR—The semi-quantitative RT-PCR method including the generation of synchronous nematode cultures for staged mRNA and subsequent cDNA synthesis are described in detail elsewhere (17). The gene combinations (fkb-3, -4, and -5 in combination with the control gene ama-1) were amplified from the cDNA samples representing staged mRNA that corresponded to two hourly intervals during post-embryonic development. The primers used were as follows: fkb-3, fkb3rtF 5′-gaggagcgga-3′ and fkb3rtR 5′-cagaatttccagcactc-3′; fkb-4, fkb4rtF 5′-cactttcaaggcgagttacgttc-3′ and fkb4rtR 5′-gagttcctcttttcctggtc-3′; fkb-5, fkb5rtF 5′-cctctagacta-tctccttcacacattc-3′ and fkb5rtR 5′-cctctagacta-tctccttcacacattc-3′. The PCR reactions were electrophoresed, Southern-blotted, and probed with fkb-3, fkb-4, or -5 PCR products labeled with [α-32P]dCTP using random priming (Prime-It® II Random Primer Labeling Kit, Stratagene), in combination with similarly labeled ama-1 PCR products. The blots were autoradiographed using a Typhoon imager (Molecular Dynamics), and bands representing the respective genes were quantified using ImageQuant 5.1 software (Molecular Dynamics). The relative abundance of the individual fkb genes were determined by comparing the signal to ama-1. At least three sets of PCR reactions with subsequent quantifications were performed to accurately determine the relative abundance of the individual fkb genes to ama-1.

Recombinant Protein Expression and PPlase Assay on FKB-3—fkb-3 was cloned from wild type C. elegans mixed stage cDNA by PCR using the following primers: FKB3F 5′-ccg-gaagttcctcttttcctggtc-3′ and FKB3R 5′-cactttcaaggcgagttacgttc-3′, digested with EcoRI and XbaI, and ligated into similarly cut pMalc2 vector (New England Biolabs) prior to transformation into XL1 expression cell lines. Maltose-binding fusion proteins were produced following the manufacturer’s instructions and then digested with factor-Xa (New England Biolabs). The affinity-purified recombinant FKB-3 was assayed for PPlase activity following published protocols (4, 18). This assay determines the rate of conversion of cis to trans of a proline-containing peptide substrate N-succinyl-ala-Leu-Pro-Phe-p-nitroanilide (Bachem). The kinetic parameters (kcat, K_m) and kcat/K_m) were calculated using spreadsheet software (Microsoft Excel).

Immunocytochemistry of C. elegans Embryos, Larvae, and Adults—DPY-7 and PH27, the respective collagen and seam cell boundary specific monoclonal antibodies were applied to freeze-cracked embryos, L1 larvae, and adults of the various strains described according to published method (19). Briefly, the washed samples were freeze-cracked on poly-L-lysine slides, blocked in 3% bovine serum albumin, washed, and the anti-DPY-7 (20) or anti-MH27 monoclonal antibodies (21) added at 1:50 dilutions. Following incubation, the slides were washed, and the anti-mouse conjugate secondary antibody Alexa Fluor 488 (Molecular probes) was applied at 1:100 dilution. Following incubation, the slides were washed and viewed by microscopy.

Microscopy—All samples were viewed either via differential interference contrast (DIC) or epifluorescence on a Zeiss Axioskop 2 microscope and images were taken with a Zeiss MrS digital camera.

RESULTS

Characterization of the Secretory Pathway-associated FKS06-binding Proteins of C. elegans—Examination of the completed genome sequence of C. elegans has uncovered eight distinct FK506-binding protein-encoding genes, termed fkb-1 to -8 (1). Of this family of proteins, FKB-3, -4, and -5 were found to possess both secretory signal peptides as assessed by SignalP and have C-terminal endoplasmic reticulum retention signals; RDEL or KEEL (Fig. 1A). In addition, these FKBs encode proteins of 27–29 kDa that have two consecutive FK506 prolyl isomerase domains separated by 25–27 amino acid linker sequences. The three FKBs also share a high level of amino acid sequence identity; 54% between FKB-3 and -4; 59% between FKB-3 and -5. The FKBs are expressed in the syncytial blastomere and thereby release the FKBs as cytoplasmic proteins. The FKBs are expressed in the syncytial blastomere and thereby release the FKBs as cytoplasmic proteins. The FKBs are expressed in the syncytial blastomere and thereby release the FKBs as cytoplasmic proteins.

FKB-3 Recombinant Protein Has Peptidyl Prolyl cis-trans Isomerase Activity—FKB-3 was cloned and expressed as a maltose-binding protein fusion, cleaved by factor-Xa and examined for PPlase activity against a synthetic Leu-Pro containing peptide substrate. The FKB-3 recombinant protein can actively convert Leu-Pro cis to Leu-Pro trans and thereby release the p-nitroanilide chromophore. The activity was calculated as: kcat = 110 ± 34 s⁻¹, K_m = 581 ± 88 mM, and kcat/K_m = 0.189 ± 0.039 × 10⁶ M⁻¹ s⁻¹, and this is comparable to previously published data for bovine FKB (kcat = 344 ± 26 s⁻¹, K_m = 520 ± 85 mM, and kcat/K_m = 0.66 ± 0.12 × 10⁶ M⁻¹ s⁻¹) (4).

FKB-3 and FKB-5 Are Expressed in the Collagen-synthesizing Hypodermis—The putative promoter regions of the fkb genes fkb-3, -4, and -5 were cloned into β-galactosidase reporter constructs in an attempt to elucidate their spatial expression patterns (regions depicted in Fig. 1B). These vectors contain a multi-intron reporter gene to enhance expression and a SV40 nuclear localization signal that aids the identification of the cells expressing the transgene. All three constructs included the translational start of the first exon and produced transgenic lines following gonadal injection. A predominantly hypodermal
cell expression pattern was displayed in the fkb-3 and -5 lines. The hypodermis is the collagen synthesizing tissue, which in turn performs many essential functions in the nematode, the most important of which being the synthesis and secretion of the cuticle exoskeleton. All lifecycle stages examined from mid-embryo through larval to adult stage displayed this hypodermal expression pattern (Fig. 2). Both fkb-3 and fkb-5 share very similar expression patterns that included the lateral seam cells, tail, and head hypodermal cells (Fig. 2). The observed expression pattern was consistent between the transgenic markers applied, namely hypodermal rol-6 and neuronal Unc-76 rescue. Transgenic lines derived from the markers applied, namely hypodermal enzymes, including dpy-18, ph-2, and pdr-2 (23). The fkb-4 transcript was detectable by RT-PCR but at a level lower than either fkb-3 or -5 (Fig. 3). A steady-state but low level of expression was observed in all larval stages and in the early adult stage but became undetectable in the mature adult stage.

Fkb Deletion Mutant Analysis Reveals a Combined Cold-sensitive Lethal Effect—Deletion mutants in fkb-3, fkb-4, and fkb-5 were screened for or were obtained from the ongoing genome-wide deletion mutant projects. The positions of the deletions were sequenced and mapped (regions depicted in Fig. 1B). Double and triple fkb deletion mutants were made by standard genetic crosses, which were confirmed by single worm PCR (result not shown). The progeny of single and combined mutants were assessed for growth and morphology defects at a range of temperatures from 12 °C to 25 °C (Table 1). Wild type, N2 strain nematodes grew slowly but otherwise developed normally at 12 °C. All single mutant strains were phenotypically similar expression patterns that included the lateral seam transcript was detectable by RT-PCR but at a level lower than either fkb-3 or -5 (Fig. 3). A steady-state but low level of expression was observed in all larval stages and in the early adult stage but became undetectable in the mature adult stage.

The Temporal Expression Pattern of the Secreted FKBs Is Consistent with a Role in Collagen Biogenesis—Semiquantitative RT-PCR was carried out to determine the expression profile of the secreted FKBs throughout the nematode post-embryonic lifecycle. The signal of the individual transcripts were measured at 2 h time points throughout larval and early adult development and were standardized by comparing them to the abundance of the constitutively expressed gene ama-1, encoding the large RNA polymerase subunit (22). These experiments were repeated in triplicate, and standard deviations were plotted for each fkb gene (Fig. 3). It is apparent from this study that fkb-3 and fkb-5 in addition to sharing a spatial expression pattern also share a similar temporal expression pattern, with both transcripts having peaks of abundance that occur at the inter-molt period for each larval stage and drop off during the actual molt and in the adult stage (Fig. 3). fkb-5 has a higher overall abundance than fkb-3 that peaks 2 h prior to fkb-3 in the earliest larval stages, namely the first and second larval stages (Fig. 3). This oscillating abundance coincides with cuticle collagen synthesis and has been observed previously for numerous cuticle collagen genes including, sqt-1, sqt-3, and dpy-13 (17) and their biosynthetic enzymes, including dpy-18, ph-2, and pdr-2 (23). The fkb-4 transcript was detectable by RT-PCR but at a level lower than either fkb-3 or -5 (Fig. 3). A steady-state but low level of expression was observed in all larval stages and in the early adult stage but became undetectable in the mature adult stage.
generate an extremely low level of Dumpy (Dpy, short and fat) mutants, but only at 12 °C. The two independent 
fkb-4(ka4), fkb-5(tm475) and 
fkb-4(ok240), fkb-5(tm475) double mutant strains (TP73 and TP86, respectively) displayed a complete larval lethal phenotype at 12 °C but remained wild type at 15–25 °C (Fig. 4). The 
fkb-3, 
fkb-4, fkb-5 triple mutant (TP83) likewise displayed a completely penetrant larval lethal phenotype at 12 °C (Table 1 and Fig. 4). The TP73, TP86, and TP83 mutants all grew slowly at 12 °C and failed to proceed beyond the first larval stage (Fig. 4). The mutant L1 larvae were shorter and slightly dumber than the wild type worms and many also exhibited a “baggy” cuticle molt defect (Fig. 4, E and F). The double (TP86) and triple (TP83) mutants also produced a range of very severe body morphology defects at 12 °C, both in the unhatched and newly hatched L1 larvae, including severe Dpy and coiled phenotypes (Fig. 7, C and E; Fig. 8, A and C).

The basis of this synthetic cold-sensitive lethal phenotype was examined in more detail by further characterizing the TP83 triple and TP86 double mutant strains. A maternal contribution to this effect was ruled out following the examination of TP83, TP86, and TP73 embryos derived from hermaphrodite mothers grown at 20 °C, then hatched and maintained on plates at 12 °C, as all strains remained larval lethal. However, when embryogenesis was completed at 15 or 20 °C and the hatched L1s were transferred to 12 °C, the TP83 and TP86 mutant strains were no longer larval lethal (Fig. 5). These mutants could now develop to adulthood, however the larval and adult stages developed slowly and were smaller in size compared with identically treated wild-type worms (compare Fig. 5, A with C and E, and B with D and F). This result confirmed that the cold-sensitive lethal effect was occurring during embryogenesis, presum-
nation with a dpy-7 allele were assessed for viability. Both transgenic lines were established and L4 stage larvae were transferred to plates at the non-permissive temperature and viable nematodes developed normally. A, adult stage wild type (N2) hermaphrodite raised at 12 °C, size is 970 μm. B, L1 stage N2 larva raised at 12 °C, sizes are 250 and 280 μm. C, TP83 (fkb-3/fkb-4/fkb-5 triple mutant) arrested L1 raised at 12 °C, size is 190 μm. D, TP83 (fkb-3/fkb-4 double) viable L2 raised at 12 °C. E, high magnification image of TP83 (fkb-3/fkb-4/fkb-5 triple mutant) arrested L1 larvae raised at 12 °C, size is 200 μm. F, high magnification image of TP86 (fkb-4/fkb-5 double) arrested L1 larvae raised at 12 °C, size is 230 μm. Scale bar in A is 100 μm; B, C, and D are 50 μm; E and F are 20 μm.

ably during the synthesis of the first larval cuticle. The ability of the TP83 triple mutant strain to enter and recover from the arrested dauer larval stage at 20 °C was also examined and found to be comparable to wild type.

Complementation of the Cold-sensitive Larval Lethal Phenotype by fkb-4 or fkb-5—The triple mutant strain TP83 and the combined fkb-4, fkb-5 double (TP86) were transformed with either a wild type copy of the fkb-4 or the fkb-5 gene in combination with a dpy-7:gfp transformation marker. Semi-stable transgenic lines were established and L4 stage larvae were transferred to plates at the non-permissive temperature and their progeny were assessed for viability. Both fkb-4 and fkb-5 were able to fully rescue the cold-sensitive larval lethal phenotype at 12 °C, with rescued nematodes developing normally through larval to egg-laying adult stages (Fig. 6). An upstream region of the adjacent C. elegans gene C50F2.5 was also removed by the fkb-5 allele tm475 and a large section of ZC455.9 coding sequence is likewise deleted by fkb-4(ok240). The involvement of these neighboring genes was excluded following transgenic rescue, as both constructs would not have reintroduced functional copies of these genes (Fig. 1B).

Analysis of the Additional Secretory Pathway FKBs—The remaining secretory pathway FKBs namely, FKB-1 and FKB-7 were analyzed to determine if they were expressed in the same tissue and if they can interact with fkb-3, -4, and -5 through reporter/promoter construct examination and via RNA interference studies, respectively. fkb-1 encodes a small 13-kDa single domain FKB that was found to be expressed exclusively in the gut, whereas fkb-7 represents a 35 kDa single domain FKB that is expressed in the nervous system (data not shown). RNA interference performed at 15 and 20 °C with either fkb-1 or fkb-7 in the fkb-triple mutant background produced no additional effects (data not shown).

Cuticle Collagen and Underlying Seam Cell Disruption in the Cold-sensitive Mutants—The cuticle and the underlying hypoderms of the double and triple mutants were examined following immunolocalization of cuticle collagen-specific antibody (DPY-7) and a seam cell boundary-specific antibody (MH27) at

| Strain | Genotype          | 12 °C  | 15 °C  | 20 °C  | 25 °C  |
|--------|-------------------|--------|--------|--------|--------|
| TP8    | fkb-3(tm348)/N    | WT     | WT     | WT     | WT     |
| Rb1213 | fkb-4(ok240)/N    | WT     | WT     | WT     | WT     |
| TP66   | fkb-4(tm475)/N    | WT     | WT     | WT     | WT     |
| TP9    | fkb-5(tm475)/N    | WT     | WT     | WT     | WT     |
| TP81   | fkb-3(tm348); fkb-4(ok240) | WT     | WT     | WT     | WT     |
| TP60   | fkb-3(tm348); fkb-5(tm475) | WT*    | WT     | WT     | WT     |
| TP73   | fkb-4(ok240); fkb-5(tm475) | Mutant | WT     | WT     | WT     |
| TP86   | fkb-3(tm348); fkb-4(ok240); fkb-5(tm475) | Mutant | WT     | WT     | WT     |
| TP83   | fkb-3(tm348); fkb-4(ok240); fkb-5(tm475) | Mutant | WT     | WT     | WT     |

* Low level of slight Dpy phenotype observed. WT, wild type.
the non-permissive temperature. In wild type embryos, the DPY-7 antibody is retained within the cell in a perinuclear ER-associated location prior to elongation. Once the first larval cuticle is laid down at elongation the DPY-7 epitope is then arranged in regular rings corresponding to the circumferential folds of the cuticle (20). This annular ring localization is found in all larval and adult stage cuticles (Fig. 7). The pre-elongated embryos of the triple (Fig. 7B) and double mutants (not shown) display the wild-type DPY-7 perinuclear localization. Following elongation the expression pattern of DPY-7 becomes extremely patchy and severely disrupted prior to (Fig. 7D) and following hatching (Fig. 7, F and H). The severity of DPY-7 disruption correlates to the severity of the morphological disruption noted in these L1 mutants (compare Fig. 7, H and F). The antibody MH27 is associated with the apical epithelial cell gap junctions and provides an excellent marker for the hypodermal seam cells that are arranged in a regular fashion along opposing lateral sides of the wild type nematode (Fig. 8H). MH27 expression is extremely disrupted and patchy in newly hatched triple and double mutants (Fig. 8, B and D) and in the arrested L1 larvae grown at 12 °C (Fig. 8F). As described for DPY-7, there is a direct correlation between the severity of morphological disruption and associated MH27 disruption. The above observations indicate that the combined fkb deletions are having affects on both the cuticle structure and the underlying hypodermis at the non-permissive temperature.

The Triple Mutant Affects the Structural Integrity of the Cuticle in a Temperature-dependent Manner—The L1 first stage larvae from the triple mutant strain were maintained at both the permissive (20 °C) and the non-permissive (12 °C) temperatures, then placed in various osmotic or reducing solutions before being assessed for the associated effects on cuticle integrity (Table 2). Wild type L1 nematodes were completely unaffected following a 10-min exposure to isotonic buffer (M9 salt solution), distilled water, or mild reducing conditions (1% β-mercaptoethanol). Likewise, TP83 triple mutants were unaffected in the isosonic M9 buffer at either temperature. Exposure to osmotic stress, in the form of distilled water resulted in 20 and 26% of the nematodes bursting at 20 and 12 °C, respectively. The mild reducing conditions of 1% β-mercaptoethanol had a significant effect at 20 °C causing 18% of the worms to
explore the secretory pathway (25). It may be hypothesized that cold-sensitive mutants include the fkb-3; -4, and -5 are differentially expressed between wild type and daf-2, dauer mutant backgrounds and confirmed that fkb-4 was expressed at an extremely low level (24). In addition to the secreted fkbS, a number of cuticle collagen genes were also found to be differentially regulated by daf-2 (24).

As would be predicted, we have demonstrated that one of the secreted FKBs has potent PPIase activity against a synthetic substrate that was comparable to FK506-binding proteins from other species (4). FKB-4 and -5 would be expected to be similarly active. The functional significance of these gene products with respect to folding the cuticle collagens and the formation of a proper cuticle was established following the examination of combined deletion mutants. A combination of deletions in all three genes or of fkb-4 and fkb-5 together led to a severe synthetic phenotype, namely a cold-sensitive lethal phenotype.

Deletion mutant embryos reared at 12 °C hatched successfully but failed to complete larval development. In addition, the mutant larvae exhibited irregular cuticle collagen expression, their underlying hypodermis was disrupted, and their cuticles were structurally weakened, being more liable to explode when exposed to mild reducing conditions. In contrast, nematodes developed normally at the permissive temperatures of 15–25 °C, exhibited normal collagen localization and, following exposure to reducing agents, displayed only a slight weakening of the cuticle. Accordingly, mutant nematodes exposed to the non-permissive temperature during their larval development are viable and predominantly wild type in appearance.

The free-living nematode C. elegans is found in anthropogenic locations throughout the world, being commonly isolated from gardens and compost heaps where it feeds on the associated microorganism-rich organic material. In these locations the predominant form is the dauer or arrested development larval stage, with mature adult stages requiring ideal environmental conditions namely food, temperature, and available oxygen. Numerous experimentally derived, temperature-sensitive mutants have been characterized in C. elegans, the majority of which are restricted at 25 °C, with cold-sensitive mutants being comparatively rare. Cold-sensitive mutants include the wrt-1 hedgehog related signaling gene, that when mutated results in embryonic and larval death in conjunction with morphological and molt defects (25). It may be hypothesized that the secretory pathway fkb genes are relatively redundant and dispensable at normal physiological temperatures (15–22 °C).

**DISCUSSION**

There are eight genes that encode FK506-binding proteins in the C. elegans genome (1), three of which encode 28–29-kDa secretory proteins with dual PPIase domains and endoplasmic reticulum retention signals. The proteins, FKB-3 and FKB-5 are spatially expressed in the collagen synthesizing hypodermal tissue, positioning them in the correct subcellular compartment for involvement in collagen biogenesis. No spatial expression pattern has so far been defined for fkb-4; however, the promoter region selected for these studies was also used to successfully rescue the cold-sensitive triple mutant, indicating that it is sufficient to generate expression but at a level undetectable in the reporter assays. The genetic interactions described between fkb-4 and -5 suggests that fkb-4 must also be expressed in the hypodermis. The temporal expression pattern confirmed that fkb-4 is expressed at a low level while fkb-3 and fkb-5 are expressed in cycles of abundance that correspond to the cuticle collagen synthesis cycle. Previous experiments have indicated that fkb-3, -4, and -5 are differentially expressed between wild type and daf-2, dauer mutant backgrounds and confirmed that fkb-4 was expressed at an extremely low level (24). In addition to the secreted fkbS, a number of cuticle collagen genes were also found to be differentially regulated by daf-2 (24).

As would be predicted, we have demonstrated that one of the secreted FKBs has potent PPIase activity against a synthetic substrate that was comparable to FK506-binding proteins from other species (4). FKB-4 and -5 would be expected to be similarly active. The functional significance of these gene products with respect to folding the cuticle collagens and the formation of a proper cuticle was established following the examination of combined deletion mutants. A combination of deletions in all three genes or of fkb-4 and fkb-5 together led to a severe synthetic phenotype, namely a cold-sensitive lethal phenotype.

Deletion mutant embryos reared at 12 °C hatched successfully but failed to complete larval development. In addition, the mutant larvae exhibited irregular cuticle collagen expression, their underlying hypodermis was disrupted, and their cuticles were structurally weakened, being more liable to explode when exposed to mild reducing conditions. In contrast, nematodes developed normally at the permissive temperatures of 15–25 °C, exhibited normal collagen localization and, following exposure to reducing agents, displayed only a slight weakening of the cuticle. Accordingly, mutant nematodes exposed to the non-permissive temperature during their larval development are viable and predominantly wild type in appearance.

The free-living nematode C. elegans is found in anthropogenic locations throughout the world, being commonly isolated from gardens and compost heaps where it feeds on the associated microorganism-rich organic material. In these locations the predominant form is the dauer or arrested development larval stage, with mature adult stages requiring ideal environmental conditions namely food, temperature, and available oxygen. Numerous experimentally derived, temperature-sensitive mutants have been characterized in C. elegans, the majority of which are restricted at 25 °C, with cold-sensitive mutants being comparatively rare. Cold-sensitive mutants include the wrt-1 hedgehog related signaling gene, that when mutated results in embryonic and larval death in conjunction with morphological and molt defects (25). It may be hypothesized that the secretory pathway fkb genes are relatively redundant and dispensable at normal physiological temperatures (15–22 °C).
but are absolutely required at subphysiological temperatures. This most probably relates to the role that FKBs play in the rate-limiting isomerization of proline bonds, in particular the proline-rich cuticle collagens that are abundantly synthesized in repeated waves that correspond to the molting cycle. It is well established that proline isomerization and proper protein folding occur in the absence of these catalysts because of thermal isomerization (4, 5, 8), and therefore removing the thermal isomerization by decreasing the physiological temperature necessitates the requirement for the PPIase activity of the FKBs. In corroboration of this, a recent study has identified a FKBP member from the psychrotrophic bacteria *Shewanella spp.* that is overexpressed at 4 °C compared with 20 °C, and it has been hypothesized to be involved in cold adaptation, aiding the proper folding of proteins at low temperatures (26).

The specific function of secretory pathway FKBs in development was previously challenged following combined *cyp* and *fkb* knock-out experiments in yeast (3). The combined immuno philin yeast knock-out was viable and the authors therefore concluded that each CYP and FK506-binding protein interacts with a unique set of proteins and performs a distinct but non-essential function (3). In metazoans this may not necessarily be the case, and there are several examples where secreted CYPs are involved in distinct functions. The best characterized example is the specific folding of the photoreceptor rhodopsin by the *ninaA* cyclophilin in *Drosophila melanogaster* (6, 7). In addition, the secreted vertebrate cyclophilin B isoform has been established as assisting the proper folding and exit of type I procollagen from the ER (27). Cyclophilin B has also been shown to accelerate the in vitro refolding of type III vertebrate collagens (8), and the inhibitor cyclosporin A effectively decreases the in vivo folding of type I procollagens (9), presumably through prolyl isomerase inhibition. In addition to having a high proline content, the non-globular collagens fold in a progressive fashion requiring each proline cis-trans isomerization to be completed before triple helix formation can proceed (8). The FKB family is by comparison less well characterized than the CYPs. FKBP65, however, represents a secretory pathway FKB that is located in the ER and is involved in ECM formation via its association with tropoelastin (10); and in addition, this PPIase has also been shown to assist in the folding of vertebrate type III collagen (28).

It can be envisaged that under normal physiological temperatures nematodes develop in a conventional manner, synthesizing waves of cuticle collagens that are in turn secreted and assembled into a complex ECM in the presence, but not the dependence, of active PPIase enzymes. Under conditions of cold stress, however, these enzymes are essential, being required to chaperone or indeed fold the collagens that must be expressed in the correct temporal, spatial, quantitative, and qualitative fashion to allow the proper folding, secretion, and assembly of the nematode cuticle components. The cuticle collagens represent 1% of the entire genome of *C. elegans*, comprising more than 170 genes (12). There is enormous complexity in the expression and the association of these collagens, which is only just beginning to be uncovered. Individual cuticle collagens can be stage-specific, and indeed there can also be additional complexity in individual collagen expression within each larval stage. It is predicted that the evolutionary conserved, ubiquitous PPIase enzymes may perform essential overlapping functions in multicellular animals including the chaperoning and catalysis of protein folding events, particularly collagen biogenesis.

Acknowledgments—The *fkb*-3(tm348) and *fkb*-5(tm475) deletion alleles were produced by the National Bio-Resource Project for the Nematode (Japan) and the *fkb*-4(ok240) deletion by the *C. elegans* Gene Knock-out Consortium (Oklahoma). The dpy-7::GFP plasmid and DPY-7 antibody were gifts from Iain Johnstone (Glasgow), the MH27 antibody was provided by Robert Waterston (St. Louis), and the Caenorhabditis Genetic Center provided some of the nematodes strains used.

REFERENCES

1. Bell, A., Monaghan, P., and Page, A. P. (2006) *Int. J. Parasitol.* 36, 261–276

2. Pemberton, T. J., and Kay, J. E. (2005) *Comp. Funct. Genomics* 6, 277–300

3. Dolinski, K., Muir, S., Cardenas, M., and Heitman, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 13093–13098

4. Kofron, J. L., Kuzmic, P., Kishore, V., Colón-Bonilla, E., and Rich, D. H. (1991) *Biochemistry* 30, 6127–6134

5. Wang, P., and Heitman, J. (2005) *Genome Biol.* 6, 226

6. Schneuwly, S., Shortridge, R. D., Larrivee, D. C., Ono, T., Ozaki, M., and Pak, W. L. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 5390–5394

7. Stammes, M. A., Shieh, B. H., Chuman, L., Harris, G. L., and Zuker, C. S. (1991) *Cell* 65, 219–227

8. Bächinger, H. P. (1987) *J. Biol. Chem.* 262, 17144–17148

9. Steinmann, B., Bruckner, P., and Superti-Furga, A. (1991) *J. Biol. Chem.* 266, 1299–1303

10. Patterson, C. E., Schaub, T., Coleman, E. J., and Davis, E. C. (2000) *Mol. Biol. Cell* 11, 3925–3935

11. Kramer, J. M. (1997) in *C. elegans II* (Riddle, D. L., Blumenthal, T., Meyer, B. J., and Priess, J. R., eds) pp. 471–500, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

12. Page, A. P., and Winter, A. D. (2003) *Adv. Parasitol.* 53, 85–148

13. Barstead, R. J. (1999) in *C. elegans: A Practical Approach* (Hope, I. A., ed) pp. 97–118, Oxford University Press, Oxford, UK

14. Brenner, S. (1974) *Genetics* 77, 71–94

15. Mello, C., and Fire, A. (1995) in *Methods in Cell Biology. Vol. 48, Caenorhabditis elegans: Modern Biological Analysis of an Organism* (Epstein, H. F., and Shakes, D. C., eds) pp. 451–482, Academic Press, San Diego

16. Fire, A. (1992) *Genetic Analysis-Biomolecular Engineering* 9, 151–158

17. Johnstone, I. L., and Barry, J. D. (1991) *J. Mol. Biol.* 219–227

18. Page, A. P., MacNiven, K., and Hengartner, M. O. (1996) *Biochem. J.* 317, 179–185

19. Rogalski, T. M., Williams, B. D., Mullen, G. P., and Moerman, D. G. (1993) *Genes Dev.* 7, 1471–1484

20. McMahon, L., Muriel, J. M., Roberts, B., Quinn, M., and Johnstone, I. L. (2003) *Biochem. J.* 378, 1366–1378

21. Francis, R., and Waterston, R. H. (1991) *J. Cell Biol.* 114, 465–479

22. Bird, D. M., and Riddle, D. L. (1989) *Mol. Cell. Biol.* 9, 4119–4130

23. Winter, A. D., and Page, A. P. (2000) *Mol. Cell. Biol.* 20, 4084–4093

24. Yu, H., and Larsen, P. L. (2001) *J. Mol. Biol.* 314, 1017–1028

25. Hao, L. M., Aspo¨ck, G., and Bu¨rglin, T. R. (2006) *Dev. Biol.* 290, 323–336

26. Suzuki, Y., Haruki, M., Takano, K., Morikawa, M., and Kanaya, S. (2004) *Eur. J. Biochem.* 271, 1372–1381

27. Smith, T., Ferreira, L. R., Hebert, C., Norris, K., and Sauk, J. J. (1995) *J. Biol. Chem.* 270, 1823–1832

28. Zeng, B. F., MacDonald, J. R., Bann, J. G., Reck, K., Gambee, J. E., Boswell, B. A., and Bachinger, H. P. (1998) *Biochem. J.* 330, 109–114