HSP25, a Small Heat Shock Protein Associated with Dense Bodies and M-lines of Body Wall Muscle in Caenorhabditis elegans*

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HSP25, a previously uncharacterized member of the α-crystallin family of small heat shock proteins in Caenorhabditis elegans, has been examined using biochemical and immunological techniques. HSP25 is the second largest of 16 identifiable small heat shock proteins in the nematode and is expressed at all developmental stages under normal growth conditions. Recombinant HSP25 produced in Escherichia coli exists predominantly as small oligomers (dimers to tetramers) and possesses chaperone activity against citrate synthase in vitro. In C. elegans, HSP25 is localized to dense bodies and M-lines in body wall muscle, to the lining of the pharynx, and to the junctions between cells of the spermathecal wall. Affinity chromatography of nematode extracts on a and to the junctions between cells of the spermathecal wall. Affinity chromatography of nematode extracts on a

The small heat shock proteins (smHSPs),† whose relationship to the α-crystallin family was first noted in Drosophila (1) and Caenorhabditis elegans (2), have been found in all kingdoms of life and, thus, have an evolutionary history dating back to the common ancestor of all present day living cells. The subunit molecular weights of smHSPs range from approximately 12,000 to 43,000, although most fall within 17,000–30,000, and generally exist as large multimeric assemblies in solution (3–5). The first three-dimensional structure determined for a smHSP, Hsp16.5, from the thermophile Methanothermobacter jannaschii, revealed a spherical array of 24 subunits of a polypeptide consisting largely of β-sheet (6). An ATP-independent chaperone activity has been demonstrated in most smHSPs studied based upon their ability to prevent the aggregation and precipitation of denatured substrate proteins (7).

In multicellular organisms, smHSPs are among the most highly induced heat shock proteins under stress conditions, and some smHSPs may also be subject to developmentally regulated expression in the absence of stress, as first noted in Drosophila studies (8). Studies on the protective effect of mammalian hsp27 revealed that cells overexpressing this protein were mainly resistant to the heat-induced disruptions seen in the microfilament lattice (9–10), and it was suggested that actin might be a major target of the protective effect of hsp27 (10). Subsequent work suggested that this protective effect was dependent on the ability of hsp27 to be phosphorylated in vivo (11). Recently, hsp27 was found to interfere with apoptosis induced by tumor necrosis factor α, probably by decreasing the level of reactive oxygen species and increasing the level of glutathione (12). This protective function was found to be dependent on the formation of large hsp27 aggregates, and in contrast to the earlier work, mutants of hsp27 in which key phosphorylation sites were eliminated also formed large aggregates and conferred protection against tumor necrosis factor α (13). The ability to form large aggregates is a property of most smHSPs, however, and is not necessarily dependent upon phosphorylation, since some members of this family are not phosphorylated (14).

Our laboratory has extensively analyzed the expression and chaperone activity of four 16-kDa smHSPs in the nematode C. elegans (2, 15–18). These smHSPs, which are strictly stress-inducible and act as molecular chaperones, likely play important roles in enhancing survival of the animal under conditions of chemical and physical stress. The completion of the C. elegans genome sequence (19) provides a unique opportunity to investigate the range of functions that members of the smHSP family carry out under normal and stress conditions in a multicellular animal. Here we describe HSP25, a novel member of the C. elegans smHSP family that possesses chaperone activity and is associated with specific structures in the body wall muscle, pharynx, and spermatheca.

MATERIALS AND METHODS

Cloning

Expression Vector pRSET23—hsp25 was amplified from first-strand cDNA with primers LD3 (5′-gat cat ATGCC ACG ACT CGA ACT-3′, forward primer, containing first ATG) or LD15 (5′-cat ATG TCG GAA CGC CGT ATC GAC-3′, forward primer, containing first ATG) or LD1 (5′-acg aag ctt CTG GAT TGC CAA TTG TGG-3′, forward primer), containing second ATG) and LD4 (5′-acg aag ctt TCA TTG CTG GAT TGC CAA-3′, reverse primer). In the primer sequences, restriction sites are underlined, and nucleotides in uppercase correspond to the genomic sequence. The amplified hsp25 gene (nucleotides 43–660 of the hsp25-coding region, Fig. 1B, derived from GenBank® sequence C09886) was cloned into the NdeI-HindIII sites of pBS23 (Invitrogen) following digestion with these enzymes.

Expression Vector pET23H6 for Expressing HSP25 with a Carboxy-terminal His$_6$ Tag (HSP25$_{His6}$)—hsp25 was subcloned from pRSET23 into the NdeI-HindIII sites of pET23a(+) (Novagen) with primers LD22 (5′-tat cca tgt gc ATG TCG GAA CGC CGT ATC-3′, forward primer) and LD23 (5′-acc aag ctt CTG GAT TGC CAA TTG TGG-3′, reverse primer).

pBS23 for Double-stranded RNA Synthesis—The hsp25 gene was digested from pET23H6 and subcloned into XbaI and XhoI sites of pBluescript-SK(+) (Stratagene).
Expression and Protein Purification of HSP25

pET23H6 was transformed into *E. coli* BL21(DE3) cells and cultured in LB medium with 25 μg/ml kanamycin, and the protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (20). Bacterial cell pellets were suspended in lysis buffer (50 mM Tris, pH 8.0, 1 mM dithiothreitol, pH 7.5) with protease inhibitor mixture (2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μM EDTA). After sonication, the cell lysate was centrifuged at 12,000 × g for 10 min, and the supernatant was passed through a DEAC column (1.5 × 5.4 cm, pre-equilibrated with TEND buffer). The unbound fraction was loaded on a Sephacryl S-300HR column (1.5 × 100 cm, 177-ml bed volume, 0.15 ml/min, 5 ml/fraction, pre-equilibrated and eluted with 50 mM Tris, 1 mM NaCl, 1 mM dithiothreitol, pH 8.0). Elution was monitored at 254 nm since HSP25 forms tryptophan residues. Fractions were analyzed by 15% SDS-PAGE (21). The purified protein was stored at 4 °C.

Expression and Protein Purification of HSP25H6

pET23H6 was transformed into *E. coli* BL21(DE3) cells and cultured in LB medium with 25 μg/ml kanamycin, and the protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (20). Bacterial cell pellets were suspended in lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, and 1% Triton X-100) with 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A. After sonication, the cell lysate was centrifuged at 12,000 × g for 10 min. The supernatant was loaded on a nickel-agarose column (nickel nitritotriacetic acid, Qiagen) at room temperature. After washing with 50 ml of lysis buffer, the column was washed with 10 ml of phosphate buffer (50 mM sodium phosphate, pH 8.0, 0.1 M NaCl) and eluted with 0.5 M imidazole in phosphate buffer, and HSP25H6 was further purified by size exclusion chromatography on a Sephacryl S-300HR column as described above.

Antibody Production

250 μg of purified recombinant HSP25 was emulsified with Complete Freund’s Adjuvant and used to immunize rabbits. Animals were boosted four times with 125 μg of HSP25 emulsified with Incomplete Freund’s Adjuvant. Antibodies were partially purified from serum by precipitation with 0.26 g/ml (NH₄)₂SO₄ followed by dialysis against phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO₄, 7.6 H₂O, 1.4 mM KH₂PO₄, pH 7.4).

Developmental Expression Profile

Synchronized N2 nematodes were cultured on NGM plates (22) at 20 °C. For heat-shock experiments, nematodes were placed on a prewarmed NGM plate at 33 °C for 2 h, then allowed to recover at 20 °C for 30 min before experiment preparation. To prepare nematode extracts, animals were suspended in 1× SDS-PAGE loading buffer (21) and heated at 100 °C for 20 min. Insoluble material was removed by centrifugation. Proteins were separated on 15% SDS-PAGE gels and transferred to an Immobilon-P membrane (Millipore) by electroblotting. Membranes were probed with anti-HSP25 antibody (1:10,000 dilution, pretreated with 1% *E. coli* acetone powder (23)) or probed with actin antibody (monoclonal anti-actin Clone 4, 1:10,000 dilution, ICN) followed by secondary antibodies (donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody, 1:10,000 dilution, Amersham Pharmacia Biotech or anti-mouse rabbit horseradish peroxidase-conjugated secondary antibody, 1:10,000 dilution, Promega). Protein-antibody complexes were detected by ECL (enhanced chemiluminescence system, Amersham Pharmacia Biotech). For comparison of different developmental stages, samples were adjusted so as to yield approximately equal signals with the anti-actin antibody.

Thermal Aggregation Assay

Thermal aggregation assays were carried out with 300 mM citrate synthase (from pig heart, Sigma) with or without HSP25 in 50 mM HEPES, pH 8.0, 25 mM NaCl, 0.5 mM dithiothreitol. Reactions were continuously monitored at 320 nm in a Cary 3E (Varian, UV-visible spectrophotometer) equipped with a thermostatted cell compartment preheated to 45 °C. The absorbance of citrate synthase alone at 40 min of heating was defined as 100% aggregation.

HSP25H6 Affinity Chromatography

To prepare an HSP25H6 affinity column, approximately 1.5 mg of HSP25H6 was loaded on a 2.0 ml nickel-agarose (Qiagen) column pre-equilibrated with lysis buffer and extensively washed with the same buffer. To prepare nematode extracts, frozen N2 nematodes (3.5 g, mixed population, non-heat-shocked, previously stored at −80 °C) were homogenized by sonication (10 s ON, 20 s OFF, total 30 min, UltraSonic Processor XL) in 20 ml of lysis buffer containing 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A. After centrifugation at 12,000 × g for 10 min, the supernatant was applied to the HSP25H6 affinity column. After washing, elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65. The elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65. The elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65. The elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65. The elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65. The elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65. The elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65. The elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65. The elution was performed with 1% acetic acid in lysis buffer. The absorbance of citrate synthase alone at 40 min was 0.65.
up to tetramers in size rather than large multimers and seem to be devoid of chaperone activity in the standard assays (29, 30).

SEC-1 is a previously studied 18-kDa smHSP with chaperone activity that is constitutively expressed and required for embryonic development in C. elegans (31). Besides C09B8.6, encoding HSP25, the remaining genes are C14F11.5 (herein named HSP43) and F52E1.7 (herein named HSP17.5). The latter have not been studied to date.

HSP25 shows a high degree of sequence identity (65%) with p27, a 27-kDa protein from the parasitic nematode Dirofilaria immitis (dog heartworm). The degree of identity is highest between the carboxyl-terminal halves of the proteins (92%). Indeed, when p27 is included in the CLUSTAL analysis with the C. elegans smHSPs, it falls closest to HSP25 (Fig. 1A, dashed line), suggesting that these proteins may be orthologs.

A number of partial cDNA clones are known for hsp25 (e.g. yk613 g9.5, yk163 h9.3, and others), indicating that this gene is expressed. However, since none of these clones extended closer to the 5′ end than codon 63, it was necessary to amplify and clone the complete cDNA by reverse transcription-polymerase chain reaction, as described under “Materials and Methods.” Several attempts to amplify cDNA using primer LD3 overlapping the methionine initiation codon (as defined in the GenBank clone C09B8.6 by GeneFinder) were unsuccessful. When primer LD15 (overlapping the second methionine codon, amino acid 15; see Fig. 1B) was used in place of LD3, a clear amplification product was detected (Fig. 2A, lane 3), and the corresponding cDNA was cloned into pRSET. Thus whether the 5′ end of the message is particularly sensitive to cleavage or whether the protein in fact begins at Met 15 remains to be determined.

When the HSP25 cDNA was expressed in E. coli, a soluble protein with an apparent molecular mass of approximately 29 kDa was obtained (Fig. 2B, lane 4). In the interest of simplicity, we will continue to refer to this protein as recombinant HSP25, although it is smaller by 1.8 kDa than that encoded by the original predicted reading frame. Size exclusion chromatography of HSP25 yielded a polydispersity profile, with most of the protein migrating between 43 and 110 kDa (Fig. 3, A and B). These data also agreed with the results of cross-linking experiments using BS3, in which the predominant species were chain reaction, as described under “Materials and Methods.”
The hsp25 gene and its product in *E. coli*. A, amplification of the hsp25 cDNA. Lane 1, DNA size marker (100-base pair [bp] ladder, Amersham Pharmacia Biotech). Lane 2, polymerase chain reaction product using primer LD3 (spanning the first ATG codon) and primer LD4. Lane 3, polymerase chain reaction product using primer LD15 (spanning the second ATG codon) and primer LD4. B, expression of pRSET23. Lanes 1 and 3, markers (ovalbumin, 44,670; carbonic anhydrase, 29,310; β-lactoglobulin, 20,190; lysozyme, 14,820). Lanes 2 and 4, total bacterial lysate before and after isopropyl-1-thio-β-D-galactopyranoside induction, respectively. Note that the apparent molecular mass of HSP25 is near 29 kDa (calculated molecular mass is 23 kDa).

The developmental expression pattern of HSP25 was examined by immunofluorescence staining. In both larvae and adults, strong staining was observed in body wall muscle (Fig. 6, A, B, D, and G) and in the lining of the pharynx (Fig. 6, J and K). When an excess of recombinant HSP25 was included in the reactions as competitor, no staining was observed (Fig. 6, M–P), indicating that the pattern seen is specific to HSP25.

The pharyngeal staining is localized to a subset of cells bordering the lumen, suggesting that these may be the marginal cells (34). These wedge-shaped cells lie at the apices of the pharyngeal lumen and contain desmosomes adjacent to the cuticle lining the lumen. Interestingly, strong HSP25 staining was also observed at the junctions between cells forming the spermathecal wall (Fig. 6L). The spermatheca is made up of 22 endothelial cells that are connected by an elaborate network of desmosomes in the spermatheca.

In body wall muscle, HSP25 was localized to a series of thin lines parallel to the long axis of the muscle fiber, alternating with thicker lines consisting of discrete spots (Fig. 6, B, D, and G). This pattern is consistent with the localization of HSP25 to the dense bodies and M-lines of the myofibrils. The dense bodies in nematode muscle are the sites of attachment of the actin or thin filaments and correspond to Z lines in vertebrate muscle. M-lines are analogous to those of vertebrate muscle...
**Fig. 6. Immunolocalization of HSP25 in C. elegans.** Nematodes were stained using rabbit polyclonal antibody to HSP25 in combination with mouse monoclonal antibodies to nematode β-integrin (MH25) or to body wall muscle M-lines (MH42) (37), followed by fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody and/or Texas Red-conjugated anti-mouse secondary antibody. A, anterior of an adult, stained with antibody to HSP25. Note the striated pattern seen in body wall muscle. B, localization of HSP25 to dense bodies (db) and M-lines (m); the dark line corresponds to a cell boundary (cb) with an adjacent muscle cell. C, anti-β-integrin also stains dense bodies and M-lines, as well as cell boundaries. D–F, co-localization of HSP25 and β-integrin. The staining patterns of HSP25 (D) and β-integrin (E) coincide in both the dense bodies
and M-lines as shown in the superimposed image, F. G–I, co-localization of HSP25 and MH42. The staining patterns of HSP25 (G) and MH42 (H) coincide in M-lines (I). J and K, pharyngeal staining pattern of HSP25 in L2 and adult, respectively. L, staining of HSP25 at junctions between cells of the spermathecal wall. M, the animal was stained with anti-HSP25 in the presence of excess recombinant HSP25 as a competitor, together with DAPI (diamidinophenylindole) to reveal cell nuclei; note the absence of detectable HSP25 signal. N, the same animal as in panel M, with staining pattern of M-lines to ensure the animals had been permeabilized. O and P, same conditions as in M and N, respectively, at a lower magnification, showing a view of the anterior of the animal.

FIG. 7. HSP25 affinity chromatography of nematode extracts. A, a nickel-agarose column was preloaded with HSP25-H6. Nematode extracts were loaded, washed, and eluted as described under “Materials and Methods.” Unbound (U) and bound (B) fractions were analyzed by SDS-PAGE and Western blotting with antibodies to HSP25, actin, vinculin, and α-actinin. Lane 1, markers; lanes 2–3, Coomassie Blue staining of unbound and bound fractions, respectively; lanes 4 and 5, Western blot detection of HSP25 and actin, respectively; lanes 6 and 7, Western blot of vinculin in unbound and bound fractions, respectively; lanes 8 and 9, Western blot of α-actinin in unbound and bound fractions, respectively. B, bound fraction from lane 3 of panel A was reloaded on a nickel column lacking HSP25-H6, and followed by SDS-PAGE and Western blotting. Lane 1, markers; lanes 2–3, Coomassie Blue staining of unbound and bound fractions, respectively; lanes 4 and 5, Western blot of vinculin in unbound and bound fractions, respectively; lanes 6 and 7, Western blot of α-actinin in unbound and bound fractions, respectively.

and arise from the stacking of the central portions of the myosin or thick filaments (36).

To further investigate the nature of the HSP25 pattern in body muscle, we compared it to the in situ pattern obtained with an antibody to the integrin β-chain, MH25 (36, 37). Integrin is localized to both dense bodies and M-lines in the nematode (37). Figs. 6, D–F, demonstrate that the patterns seen within the myofibrils with anti-HSP25 and MH25 are superimposable, confirming the localization of HSP25 to the dense bodies and M-lines. The anti-integrin antibody, however, also stained the junctions between individual muscle cells (38), which was not the case with anti-HSP25 (compare Fig. 6, B and C). As further confirmation of HSP25 localization, MH42, a monoclonal antibody that stains the M-lines of nematode muscle (37) was found to localize to the thin continuous lines seen in the HSP25 stained muscle cells (Fig. 6, G–I).

To investigate the possibility that HSP25 might interact with specific components in dense bodies, recombinant HSP25 carrying a carboxyl-terminal histidine tag (HSP25-H6) was bound to a nickel affinity resin and used as an affinity ligand. Extracts prepared from adult nematodes were applied to the column, and tightly bound proteins were eluted with 4 M urea. Extracts prepared from adult nematodes were applied to the column, and tightly bound proteins were eluted with 4 M urea. Unbound (U) and bound (B) fractions were analyzed by SDS-PAGE and Western blotting with antibodies to various dense body components (Fig. 7). Coomassie Blue staining of the bound proteins (Fig. 7A, lanes 2 and 3) revealed a prominent band with an apparent molecular weight near 29,000 and a series of faint bands at higher molecular weights. When the bound and unbound fractions were probed by Western blotting with antibodies to actin and HSP25 (Fig. 7A, lanes 4 and 5), the 29-kDa band was confirmed as HSP25. The HSP25 eluted from the column likely resulted from the formation of mixed oligomers between the immobilized HSP25 and HSP25 in the extract. Actin was detected in the unbound fraction but not in the bound fraction.

In contrast to actin, Western blots of HSP25 column fractions probed with monoclonal antibodies to vinculin or α-actinin revealed that a large fraction of these proteins had bound to the column (Fig. 7A, lanes 7–9). Lower molecular weight bands detected in the bound fractions using these antibodies likely represent degradation products of vinculin and α-actinin. When the bound proteins from the HSP25 column were re-applied to a nickel affinity resin lacking HSP25, vinculin and α-actinin failed to bind, suggesting that the interaction is specific for the HSP25 ligand (Fig. 7B, lanes 4–7).

Double-stranded RNA can act as a signal for gene-specific silencing of expression in C. elegans (25, 35). Injection of double-stranded RNA corresponding to the coding region of a gene results in potent and specific interference with the expression of that gene. The silencing effect is seen in the injected animal and its F1 progeny and often mimics the null phenotype of the gene in question. To examine the possible phenotype of an HSP25 knock-out mutation, we therefore carried out RNAi experiments using double-stranded RNA made from the HSP25 gene (nucleotides 43–660). Under conditions that produced suppression of a myosin-GFP fusion gene in approximately 90% of the progeny of injected animals, no effect of HSP25 RNAi on embryo viability was seen, and the progeny of the injected nematodes developed normally (data not shown).

DISCUSSION

The α-crystallin family of smHSPs are ubiquitous molecular chaperones in prokaryotic and eukaryotic cells (3–5). In vitro, these proteins act as general inhibitors of protein aggregation and precipitation (7). Although they are incapable of catalyzing the refolding of polypeptide substrates, evidence suggests that smHSPs are able to bind partially denatured proteins and hold them in a folding competent state for interaction with other chaperones which may catalyze refolding (39–41).

The availability of the complete genome sequence for C. elegans (19) provides a unique opportunity to examine the full scope of small HSP gene structure and function in an animal model. A search of the nematode genome for members of this family revealed 16 genes (Fig. 1A). Previous studies have shown that the 16-kDa smHSPs are strictly stress-inducible (15–16, 42) and likely function to prevent protein denaturation...
under adverse cellular conditions. Other smHSPs, such as SEC-1 (31) and the four 12-kDa smHSPs are produced under normal (i.e. non-stress) conditions at specific stages during C. elegans development (29, 30). This suggests that they may perform specialized functions within certain tissues, or they may interact with specific classes of protein substrates. The studies presented here indicate that HSP25 is localized to the dense bodies and M-lines of the sarcosome within body wall muscle. This is the first demonstration of specific subcellular localization of a C. elegans small heat shock protein. The dense bodies in nematode muscle cells, which are analogous to the focal adhesion plaques of vertebrate non-muscle cells and the dense plaques of smooth muscle (36), are complex structures containing the proteins β-integrin (36–37), vinculin (43), α-actinin (36, 44), talin (45), and actin (46). In addition to the immunolocalization of HSP25 to dense bodies and M-lines, vinculin and α-actinin were found to bind to an HSP25 affinity column, further supporting a functional interaction between these proteins. The binding of both vinculin and α-actinin, two proteins normally associated in vivo with dense body structures, to HSP25 in vitro and the lack of binding of a much more abundant protein, actin, suggest that the interaction observed biochemically reflects a physiological role. The assembly of a focal adhesion plaque requires the coordinated recruitment of β-integrin, vinculin, α-actinin, talin, actin, and perhaps other proteins to specific membrane sites (reviewed in Ref. 45), and the co-localization of a smHSP to these sites in nematode body wall muscle suggests that HSP25 may be involved in the maintenance, turnover, or assembly of focal adhesion structures. Other possible roles for HSP25 might be as a general chaperone associated with muscle protein turnover or in the maintenance of preformed structures within muscle cells. It has been shown recently that degradation of a major fraction of vertebrate muscle proteins occurs via the ubiquitin-dependent proteasome pathway (47), and the degradation of muscle proteins during programmed cell death in the hawk moth, Manduca, occurs via the ubiquitin-dependent system (48). Proteasome inhibition can result in activation of heat shock transcription factor 2 in mammalian cells (49), and of all members of the heat shock transcription factor family in avian cells (50), resulting in the induction of all classes of HSPs, including the smHSPs. In this context a loss of HSP25 might not lead to a discernible phenotype in the absence of other stresses or of large scale muscle protein turnover. Consistent with this hypothesis is the finding that another member of the smHSP family, MKBP, binds and activates the myotonic dystrophy protein kinase, MDPK (51). Although this chaperone has been shown in vitro to protect MDPK from heat-induced inactivation, the smHSP itself is not heat-induced in muscle but rather is constitutively expressed. This is consistent with the fact that muscle cells are frequently and rapidly subjected to severe heat and oxidative and mechanical stresses, so that the continued presence of smHSP chaperones might be advantageous.

In this view, it is possible that the localization sites of HSP25 observed here may represent storage sites from which the active chaperone can be recruited during stress. Indeed, at present we cannot rule out the possibility that the vinculin and α-actinin recognized in vitro by HSP25 may have been partially unfolded, and the lack of interaction with actin might indicate that actin is simply more stable under the isolation conditions used. At least three possibilities may be envisaged for the lack of effect seen in hsp25 RNAi experiments. First, it is conceivable that up-regulation of another member of the smHSP family might have compensated for a decrease in HSP25. Second, the phenotype of an HSP25 deficiency might be apparent only under specific physiological conditions. Finally, given the present state of knowledge regarding the mechanism of RNAi effects, we cannot rule out the possibility that the hsp25 gene may be relatively resistant to RNA-mediated interference. The isolation of an authentic hsp25 genetic null should allow discrimination among these alternatives.

A 27-kDa smHSP, p27, from the mammalian parasitic nematode D. immitis is closely related to C. elegans HSP25 (Fig. 1A). This protein was also found to be constitutively expressed, and immuno-electron microscopy showed that antibodies to recombinant p27 bound to the region immediately adjacent to the hypodermal membrane on the cytoplasmic side of L3 and L4 larvae in Dirofilaria (52). The dense bodies of body wall muscle cells attach through integrins to the extracellular matrix and, hence, to the overlying hypodermis (36). Thus the localization of D. immitis p27 is consistent with that of HSP25 in C. elegans at the current level of resolution, suggesting that these proteins may perform closely related functions in different nematode species.

As noted above, smHSPs containing bound substrate proteins have been shown to interact with other chaperones, resulting in the catalysis of protein refolding. Taken together, these observations and our results provide support for the involvement of molecular chaperones and specifically for smHSPs in the maintenance and/or disassembly of components of C. elegans body wall muscle. A combination of genetic and molecular approaches, readily available in C. elegans, will be required to elucidate the precise roles of HSP25 in these processes.

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