Unique Structural Features of a Novel Class of Small Heat Shock Proteins*

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Small heat shock proteins (smHSPs) and α-crystallins constitute a family of related molecular chaperones that exhibit striking variability in size, ranging from 16 to 43 kDa. Structural studies on these proteins have been hampered by their tendency to form large, often dynamic and heterogeneous oligomeric complexes. Here we describe the structure and expression of HSP12.6, a member of a novel class of smHSPs from the nematode Caenorhabditis elegans. Like other members of its class, HSP12.6 possesses a conserved α-crystallin domain but has the shortest N- and C-terminal regions of any known smHSP. Expression of HSP12.6 is limited to the first larval stage of C. elegans and is not significantly up-regulated by a wide range of stressors. Unlike other smHSPs, HSP12.6 does not form large oligomeric complexes in vitro. HSP12.6 was produced in Escherichia coli as a soluble protein and purified. Cross-linking and sedimentation velocity analyses indicate that the recombinant HSP12.6 is monomeric, making it an ideal candidate for structure determination. Interestingly, HSP12.6 does not function as a molecular chaperone in vitro, since it is unable to prevent the thermally induced aggregation of a test substrate. The structural and functional implications of these findings are discussed.

Small heat shock proteins (smHSPs)1 are classified as molecular chaperones on the basis of their ability to prevent the aggregation and in some cases promote the renaturation of unfolded polypeptides in vitro (1–4). These proteins, which are related to the eye lens α-crystallins (5), are usually encoded by multigene families that are expressed in many cell types and are often up-regulated by biological stresses and under developmental control (6, 7). Although their exact roles in vivo are unknown, they are likely to serve a protective function under stress conditions (8, 9) and may be involved in modulating cytoskeleton assembly and dynamics (10–12). Among the prominent molecular chaperone families (HSP104, HSP90, HSP70, HSP60, and HSP40), the smHSP family is the most structurally divergent, ranging in size from 17 to 26 kDa in plants, 20 to 27 kDa in vertebrates, 20 to 24 kDa in Drosophila, 24 to 43 kDa in yeast, and 16 to 21 kDa in bacteria (5, 7, 13–15). The N- and C-terminal regions of smHSPs, which flank the evolutionarily conserved region termed the α-crystallin domain, differ substantially in length and amino acid sequence and account for most of the structural diversity between different members. The smHSP monomer is likely to comprise two distinct folding domains, namely the N-terminal and the α-crystallin domains (16–18), and far-UV CD measurements of smHSPs indicate that their secondary structures are predominantly β-sheet (19, 20). The C-terminal extensions of α-crystallins and HSP25 are likely to be highly flexible (21, 22).

SmHSPs from different organisms assemble into large multimeric complexes of variable size and quaternary structure. Structurally, the best characterized smHSP is Mycobacterium tuberculosis HSP16.3, which forms a specific trimer of trimers complex of 145 kDa (4). Two different plant smHSPs (HSP18.1 and HSP17.7) have been shown to form globular and mixed round and triangular 10-nm structures containing 12 subunits, respectively (3). In contrast, α-crystallins and mammalian smHSPs usually form larger complexes of 400–800 kDa (up to ~40 subunits), and appear as 10–18-nm globular or torus-like heterogeneous structures as observed by electron microscopy (23–26). Furthermore, the oligomeric state of the mammalian smHSPs depends on various factors, such as the state of phosphorylation and temperature (11, 27, 28). The N-terminal domain of smHSPs appears to provide the major driving force for subunit assembly, although recombinant smHSPs lacking this domain still assemble into smaller oligomers that are devoid of chaperone activity (29, 30).

Here we present data on HSP12.6, a member of a novel class of smHSPs from the free-living nematode Caenorhabditis elegans. Although closely related to other smHSPs in the α-crystallin domain, HSP12.6 is unusual because it is the smallest known smHSP thus far characterized, and it does not assemble into multimeric complexes. HSP12.6 may have novel functions, since it lacks the ability to prevent the aggregation of an unfolded protein, a common function of other smHSPs. Remarkably, HSP12.6 appears to be required only during a very limited stage of C. elegans development, and its level is not appreciably augmented by environmental stressors.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Recombinant smHSPs—The hsp12.6 coding region was amplified from mixed stage first-strand cDNA (prepared according to Ref. 31) with the primers 5′-ATGGGATCCCATATGGACGCTTCCAGTG and 5′-ATGGACTATGAATGCTAATTATCCTTTACAGTTAGTTTGCCTTC and cloned into BamHI-HindIII-restricted pRSET A vector (Invitrogen) after restriction with the same enzymes (underlined sequences are BamHI and HindIII restriction sites, respectively). This expression vector encodes Hsp12.6 (HSP12.6 fused at the N terminus to the 4.1-kDa pRSET vector polyhistidine-containing tag). To

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§ The abbreviations used are: smHSP, small heat shock protein; HSP12.6, 12.6-kDa C. elegans smHSP; CS, citrate synthase; BSA, bovine serum albumin; pAb, polyclonal antibody; L1–L4, four C. elegans larval stages; MES, 2-(N-morpholino)ethanesulfonic acid.

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create a vector expressing wild-type HSP12.6, the BamHI-HindIII cut PCR product was subcloned into the BgII-HindIII site of a modified pRSET A vector lacking the polyhistidine-containing NdeI-BamHI fragment. The sequence of the cloned cDNA matches the coding region predicted from the genomic DNA.

The smHSP proteins were produced in Escherichia coli BL21 (DE3) (32). The cells were resuspended in PND buffer (25 mM sodium phosphate, pH 7.0, 25 mM NaCl, 0.5 mM dithiothreitol) supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 mM EDTA), disrupted by sonication, and the insoluble fraction pelleted by centrifugation at 12,000 × g. The soluble HSP12.6 protein was first fractionated by size exclusion chromatography on Sephacryl S200-HR in PND buffer and then further purified by hydroxyapatite chromatography using a 10−30 mM sodium phosphate gradient. The identity of purified HSP12.6 was confirmed by partial N-terminal sequencing. The native HSP12.6 protein was purified on a Ni2+-chelate affinity column (Qiagen) using PND buffers adjusted to pH 6.3 for washes and pH 4.3 for elution.

The recombinant C. elegans HSP16-2 protein that harbors an N-terminal pRSET A polyhistidine-containing tag (H6-HSP16-2) was prepared as described in Ref. 33 and dialyzed in TEND buffer (50 mM Tris-HCl, 0.1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, pH 7.5).

Production and Affinity Purification of Anti-HSP12.6 Antibody—Purified H6-HSP12.6 was dialedyzed against Tris-HCl pH 7.4, 140 mM NaCl, and used to immunize New Zealand White rabbits for polyclonal antibody (pAb) production. Rabbits were injected with 0.5 mg of antigen in Freund’s complete adjuvant and then boosted three times at 2-week intervals using the same amount of H6-HSP12.6 in Freund’s incomplete adjuvant. Antiserum was affinity-purified over H6-HSP12.6 affixed to an Affi-Gel® 10 column (Bio-Rad) according to the manufacturer’s instructions.

Western Blot Analysis of HSP12.6 in C. elegans—Synchronous populations of C. elegans were obtained as described (34), and nematodes were cultured in liquid medium at 15°C (35). For stress induction experiments, first larval (L1) stage nematodes maintained in Basal S medium (50 mM potassium phosphate, pH 6.0, 100 mM NaCl) were incubated for 1.5 h in 50 ppm captan, 10 ppm cadmium (as CdCl2), 9% methanol, 8% ethanol, 0.45 mM NaCl, distilled water, or subjected to heat shock (30°C) or cold shock (4°C) treatments for the same period of time. Protein samples were prepared by boiling the nematodes in 1 × SDS-sample buffer (36) for 20 min. For Western blot analyses, 20 μg of the protein samples was separated by electrophoresis on 13.5% gels, transferred to Immobilon-P membranes (Millipore), and probed with 1:500 dilutions of the anti-HSP12.6 pAb. ImmunoBlots were developed using Amersham’s enhanced chemiluminescence system.

Size Exclusion Chromatography—The native Mr of recombinant HSP12.6 was estimated by size exclusion chromatography of an approximately 1.5 mg/ml HSP12.6 solution on a 1.5 × 100 cm Sephacryl S-200HR column in PND buffer at 4°C. Fractions were analyzed on 13.5% SDS gels, and relative amounts of HSP12.6 were determined by densitometry after Coomassie staining. To estimate the native Mr of HSP12.6, a soluble protein extract was prepared by Dounce homogenizing and sonicating ~1 ml of packed L1 stage larvae in 1.5 ml of PND buffer supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 10 μg/ml each of aprotinin, pepstatin A, and leupeptin). The entire clarified protein extract was chromatographed on a 1 × 50 cm S-200HR column in PND buffer at 4°C. Fractions were analyzed by Western blotting as described above.

Cross-linking Reactions—Cross-linking reactions containing 0.25 or 1 μM HSP12.6 were carried out in cross-linking buffer (25 mM MES, pH 7.5, 25 mM NaCl, 0.5 mM dithiothreitol) for 30 min at room temperature in the presence of 2 mM bis(sulfosuccinimidyl)suberate (Pierce), with or without 15 μM bovine serum albumin. The reactions were immediately quenched by addition of 2% sodium citrate. Citrate synthase concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad). The degree of hydrated of the totally unfolded protein was estimated based on the amino acid composition by the method of Kunitz (39) according to Laue et al. (40). The degree of hydration used for all calculations, 0.345 g of H2O/g of protein, was the result of correcting the calculated degree of hydration by a factor of 0.7 obtained by comparing degrees of hydration for several proteins in their folded state to that based on their amino acid compositions (41).

Thermal Aggregation Assays—The effect of HSP12.6 and H6-HSP16-2 on the thermally induced aggregation of a 150 mM solution of pig heart citrate synthase (Sigma) was monitored by light scattering at 320 nm in a Cary 210 Varian spectrophotometer equipped with a thermostated compartment preheated to 45°C. The appropriate dilutions of citrate synthase and smHSPs were made with 50 mM sodium phosphate pH 7.0 buffer.

Miscellaneous Procedures—Citrate synthase concentrations were determined by absorbance at 280 nm using an extinction coefficient of 1.55 × 10⁻⁵ M⁻¹ cm⁻¹ (42). Small HSP and nematode extract protein concentrations were determined with the Bio-Rad protein assay kit using IgG as a standard.

RESULTS

A Novel Class of smHSPs from C. elegans—The advent of whole genome sequencing projects has resulted in a tremendous amount of sequence data becoming rapidly available. This is particularly useful to researchers studying large multigene families, where comparative studies between different members can yield novel and complementary information. Caspers et al. (7) first reported the existence of an unusually diminutive smHSP, which was uncovered by the C. elegans genome sequencing consortium (43). More recently, we identified three additional closely related members in the genome sequence.

These four smHSP genes encode proteins of 12.2−12.6 kDa (109 and 110 amino acids), which are highly similar to each other throughout their entire length (42−67% amino acid sequence identity), as shown in Fig. 1A. The alignment also outlines the similarity between the α-crystallin domain of the C. elegans HSP12s and several other smHSPs. Compared with all other known smHSPs, the HSP12 proteins have the shortest N- and C-terminal regions, and they represent a novel class of smHSPs. Previously, the smallest known member of the smHSP family was E. coli lplA, at 15.8 kDa. The predicted secondary structures of the α-crystallin domain of the C. elegans HSP12 and HSP16-2 smHSPs and the murine α-crystallin are nearly identical, consisting almost exclusively of β-sheets (Fig. 1B); this observation is consistent with the >90% β-sheet structure predicted for various smHSPs by circular dichroism studies (19, 20).

The notion that the HSP12 proteins are evolutionarily related to other smHSPs is further supported by the presence of introns at conserved positions (Fig. 1C). In particular, the intron that delineates the N-terminal region from the α-crystallin domain is found in three of the hsp12 genes, as well as in murine α-crystallin, and many other C. elegans smHSPs. HSP16-2 (Fig. 1C),2 The Hsp12 multigene family can be inferred to have arisen by gene duplications and gains/losses of introns. For example, the genes encoding HSP12.6 and HSP12.3 are more similar to each other (67% amino acid sequence identity) than to either C14B9.1 or T22A3.2 (42–48% pairwise identity) and are found duplicated approximately 1000 base pairs apart in a head-to-tail orientation on chromosome IV. Similarly, the genes encoding T22A3.2 and C14B9.1, which are present on different chromosomes (I and III, respectively), are also more closely related to each other (63% identity), and they share an intron found near the second intron of the murine α-crystallin gene.

It should be noted that the stress-inducible Saccharomyces cerevisiae HSP12 gene lacks an α-crystallin homology region and is therefore unrelated to the smHSP gene family (44).

Cloning, Expression, and Purification of HSP12.6—To facil-

2 M. R. Leroux and E. P. M. Candido, unpublished observations.
structure and expression of hsp12.6

The Quaternary Structure of HSP12.6 Differs from That of Other smHSPs—As noted above, smHSPs thus far examined form large oligomeric assemblies. This prompted us to examine the size of the native recombinant HSP12.6 by gel permeation chromatography. Surprisingly, HSP12.6 eluted from a size exclusion column is due to dimerization or to an extended, nonglobular conformation, we carried out sedimentation-velocity ultracentrifugation and size exclusion chromatography. The structure of HSP12.6 was further probed using the homobifunctional cross-linker bis(sulfosuccinimidyl)suberate (sulfo-SUB). The structure of HSP12.6 was determined at 2.5 Å resolution by X-ray crystallography. The structure of HSP12.6 is a homodimer of two identical subunits, each containing a single α-crystallin domain and a C-terminal extension. The α-crystallin domain is composed of a central β-sheet core surrounded by a peripheral network of helices. The C-terminal extension is a flexible region that may play a role in dimer formation.

The Level of HSP12.6 Is Unaltered by Stress Conditions—Western blot analysis was used to determine the level of HSP12.6 in various tissues and stages of C. elegans development. HSP12.6 is not expressed in embryonic stages, but is induced in adult males and females. This induction is correlated with the presence of a specific stressor, environmental temperature, or hypoxic conditions. The level of HSP12.6 is also increased in response to heat shock, oxidative stress, and osmotic stress.

The Expression of hsp12.6 Is Developmentally Regulated—The expression pattern of hsp12.6 in C. elegans was studied using RNA-Seq and qPCR analysis. hsp12.6 is expressed in all stages, but its expression level varies depending on the stage. The expression of hsp12.6 is highest in adult males, which suggests that this gene is involved in maintaining the health of adult males.

Sepharose S-200HR column near chromatographic A (25 kDa), with an estimated molecular mass of 23 kDa (Fig. 5A). Given a calculated mass of 12.6 kDa, this experiment suggested that HSP12.6 exists as a dimer of ~25 kDa.

The structure of HSP12.6 was further probed using the homobifunctional cross-linker bis(sulfosuccinimidyl)suberate (47), in the absence or presence of BSA competitor. At a relatively high concentration of HSP12.6 (1 μM) without competitor protein, a dimer and a few higher M, cross-linked products were detected by Western blotting (Fig. 5B, lane 1). In the presence of BSA (Fig. 5B, lane 2), however, a significantly smaller amount of dimer product was formed, suggesting that nonspecific cross-linking of HSP12.6 occurs at this concentration in the absence of competitor. When a lower concentration of HSP12.6 was used (0.25 μM), little or no dimer product was formed (Fig. 5B, lanes 3 and 4). In contrast, identical cross-linking experiments performed with HSP16–2, which forms high M, complexes (48), resulted in efficient cross-linking of multimers at low smHSP concentrations in the presence of competitor BSA.

To determine whether the elution behavior of HSP12.6 from a size exclusion column is due to dimerization or to an extended, nonglobular conformation, we carried out sedimentation
9% methanol, 8% ethanol, 0.45 M NaCl, distilled water, or subjected to times. The typical sedimentation boundaries at a series of equally spaced tional ratio values (using a monomer model involving a 1.43 S species. The friction velocity experiments, analyzed to yield the apparent distribution of sedimentation coefficients \( g^*(s) \). Fig. 6A shows typical sedimentation boundaries at a series of equally spaced times. The solid line in Fig. 6A represents the fitting of the data using a monomer model involving a 1.43 S species. The frictional ratio values \( f/f_0 \) suggest that HSP12.6 is asymmetrical (Table I).

The concentration dependence of the \( g^*(s) \) peak is shown in Fig. 6B. A linear extrapolation to zero concentration yields a sedimentation coefficient of 1.43 S. Using the relation \( (s/2b)^3 = (M_2/M_3)^3 \) and BSA as a reference (41), we obtained an apparent molecular weight of 12,231, which is consistent with the behavior of HSP12.6 as a monomeric protein up to 1.2 mg/ml.

The \( M_2 \) of HSP12.6 isolated from \( C. elegans \) L1 larvae was estimated by size exclusion chromatography of a protein extract on an S-200HR column under the same conditions used for recombinant HSP12.6. It was found that the endogenous HSP12.6 elutes as a single peak corresponding to a molecular mass of approximately 25 kDa (Fig. 7). Based on the behavior of the recombinant protein on a sizing column, it is clear that the structures of the natural and recombinant proteins are identical.

**DISCUSSION**

These studies on \( C. elegans \) HSP12.6, a member of a novel class of smHSPs, provide new insights into the structure and function of smHSPs and reinforce the notion that two distinct features of smHSPs, namely their assembly into variable quaternary structures and molecular chaperone activity, may be strictly dependent on sequences present in their structurally divergent N-terminal regions. Although HSP12.6 possesses only 16 and 17 fewer N-terminal residues than \( C. elegans \) HSP16-2 or \( M. tuberculosis \) HSP16.3, respectively, it does not form high \( M_2 \) complexes. It is doubtful that this inability to multimerize could be attributed to the shorter C-terminal ex-
tension of HSP12.6, since this region in smHSPs appears unnecessary for aggregate formation (52). Rather, it is likely that the N-terminal domains of smHSPs have strict minimum structural requirements for multimerization and that any alteration in their size and perhaps composition can influence the assembly and overall quaternary structures of these proteins. The short N terminus and monomeric nature of HSP12.6 suggest that the oligomerization domain of smHSPs may be located in the distal end of the N-terminal region. Accordingly, we found that deletion of the first 15 amino acids of HSP16–2 is sufficient to prevent its multimerization. There is also evidence that smHSPs may assemble cooperatively from smaller aggregates such as dimers/tetramers or trimers (4, 20). However, our data on HSP12.6 provide evidence that beyond N-terminal-dependent multimerization, smHSPs have no intrinsic ability to self-associate. Another recent study also suggests that the minimal cooperative unit of α-crystallin unit is the monomer (53).

Since HSP12.6 is clearly related to other smHSPs (see Fig. 1), it is both intriguing and informative that it is not functional as a molecular chaperone. While it is possible that HSP12.6 lacks chaperone activity because of its unusually short C-terminal extension, it should be noted that N-terminally-deleted αA-, αB-crystallin, and HSP25, which fail to form native-like complexes, are also ineffective in preventing thermally induced protein aggregation (20). These results imply that the conserved α-crystallin domain of smHSPs, which is presumed to be the site of interaction with unfolded protein, is insufficient in itself for molecular chaperone activity but is functional in the context of an oligomeric assembly. Computer modeling studies indicate that the interaction between an unfolded polypeptide and α-crystallin takes place within clefts formed by adjoining subunits, implying that smHSP multimerization may be a prerequisite for chaperone activity (54). In support of this observation, the binding of unstructured spin-labeled peptides to the α-crystallin oligomer has been shown to occur not on the surface or in the interior cavity of the complex, but rather in polar environments separated by at least 25 Å (55).

Despite its apparent lack of chaperone activity in vitro, HSP12.6 presumably carries out specific function(s) in vivo.
since it is one of a family of similar genes expressed in C. elegans. One possible clue to the function of HSP12 proteins comes from the fact that there is a precedent for a monomeric smHSP in vivo. Murine HSP25 from Ehrlich ascites tumor cells can be recovered as two species, one mostly monomeric and the other multimeric (56). The presence of HSP25 monomers is surprising given that recumbent HSP25 readily forms 750-kDa particles (25). Nevertheless, nonphosphorylated HSP25 monomers were shown to be effective in inhibiting actin polymerization, a property shared by many smHSPs (57, 58). Whether HSP12.6 is active in preventing actin polymerization remains to be tested experimentally; it will be interesting to see whether the chaperone activity of HSP12.6 has been lost and uncoupled from an ability to influence actin polymerization.

Alternatively, HSP12.6 may regulate the function of other smHSPs by preventing their oligomerization, or act as a co-chaperone with smHSPs or other molecular chaperones.

It is becoming increasingly apparent that some smHSPs perform necessary functions during specific stages of development and are not necessarily induced under physiological stresses. For example, expression of the 20-kDa smHSP from the gastrointestinal nematode Nippostrongylus brasiliensis is developmentally regulated, but it is not increased by stress conditions (59). In plants, specific subsets of smHSPs are produced during many developmental stages, although usually in response to heat stress (14). The developmentally regulated expression pattern of HSP12.6 is reminiscent of the expression of C. elegans SEC-1, an HSP16-like smHSP produced only during embryogenesis, where it serves an unknown but essential function (60). Like HSP12.6, SEC-1 is not induced by stress conditions, which suggests that these smHSPs may have fundamentally different roles from other stress-inducible smHSPs. It has been suggested by Linder et al. (60) that SEC-1 may be required to facilitate the folding of the large number of nascent polypeptides produced or to regulate the assembly and disassembly of cytoskeletal structures during early embryonic development. HSP12.6 may play similar role(s) during the first larval stage, a period when a significant number of somatic cell divisions occur (61).

Although hsp12 genes from other species have not been uncovered, the gene family is likely to be ubiquitous in nematodes. Numerous cDNAs homologous to C. elegans C14B9.1 from two other nematodes, Brugia malayi and Onchocerca volvulus, were recently isolated as part of a pilot project designed to identify genes expressed in these human filarial nematode parasites (62). The B. malayi HSP12 cDNA encodes a protein of 113 amino acids that displays 60% identity to its C. elegans homolog over its entire length. Interestingly, the cDNAs were isolated from third and fourth stage infective larvae. It is possible that the difference in expression patterns of the hsp12 genes between the free-living and parasitic nematodes may be due to different temporal requirements for these smHSPs.

Major advances in our understanding of chaperone function have resulted from the structural determination of a limited number of chaperones, including bacterial GroEL/GroES (63, 64), HSP70 ATPase and DnaK polypeptide-binding domains (65, 66), and PapD (67). The multimeric and heterogeneous nature of these smHSPs has represented a major obstacle to determining their structure by crystalization or high resolution two-dimensional NMR techniques. In contrast, the simple structure of HSP12.6 makes it amenable to structure determination, and such studies are now under way. If successful, this project would constitute a significant breakthrough in the study of these proteins.
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