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Studies of the small heat shock proteins of *Caenorhabditis elegans* using anti-peptide antibodies

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Peptides corresponding to selected regions of the 16 kDa small heat shock proteins (hsps) of the nematode *C. elegans* were synthesized and used to elicit polyclonal antibodies. It was found that these antibodies reacted predominantly with either the 16 kDa or the 18 kDa proteins, suggesting a close structural similarity between these hsps. Western blots of two-dimensional gels revealed extensive heterogeneity in these proteins, probably resulting from post-synthetic modifications. The native structures of both size classes of hsps were found to consist of large complexes of 4–5 × 10^5 Da.

Heat shock protein; *Caenorhabditis elegans*; α-Crystallin; Aggregation

1. INTRODUCTION

Eukaryotic cells respond to slight increases in ambient temperature by synthesizing a number of distinct proteins, termed heat shock proteins (hsps). These proteins may be grouped into several families based on sequence similarities: the hsp70-grp78 family, the hsp83-90 family, the hsp60 family and the low molecular weight or small hsps, which range in molecular mass from approximately 16-kDa [1].

The low molecular weight or small hsps possess a region of extensive homology to the α-crystallins of the vertebrate lens [2]. Constitutive expression of the 27 kDa human hsp in rodent cells conferred a thermoresistant phenotype on the latter [3], so it is likely that these hsps play an important role in determining cellular resistance to heat shock and to other stresses which induce them. The small hsps of *Drosophila* and vertebrates have been found to exist as large cytoplasmic aggregates [4]. In the nematode *C. elegans*, the major low molecular weight hsps have apparent masses, based on SDS-PAGE, of 16 and 18 kDa, or approximately 10 kDa smaller in size than the related vertebrate or *Drosophila* proteins [1,5]. We have previously cloned and characterized the genes encoding the 16 kDa hsps of *C. elegans* [6,7]. The nematode small hsps, like their counterparts in other eukaryotes, possess a region of homology with the α-crystallins which extends over approximately 72 amino acid residues [5]. Here we report the results of imunological and biochemical studies on the hsp16 proteins which indicate that they exist in the native form as high molecular weight aggregates. The hsp16 and hsp18 are found to be structurally related, and both classes of small hsp consist of multiple isoforms, suggesting that they may be subject to post-translational modifications.

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2. MATERIALS AND METHODS

2.1. *Culture of* C. elegans *and heat shock conditions*

Caenorhabditis elegans Bristol N2 strain was grown on NGM plates as described by Brenner [8]. Liquid cultures from 0.25 liters to 25 liters (commonly 4 liters) were grown essentially according to the procedure of Sulston and Brenner [9], except that commercially-grown *E. coli* (strain MRE 600 obtained washed and frozen from Grain Processing Corporation, Muscatine, Iowa) was used as a food source. Embryos were prepared from gravid adults by alkaline hypochlorite treatment [10].

Embryos were heat-shocked either on NGM plates or in liquid culture. For the liquid culture, embryos were added to Basal S medium in a baffled Erlenmyer flask and shaken in a water bath. In either case, the heat shock was for 2 h at 33°C and was usually followed by a recovery period at 18–20°C of 8–12 h, and a second 2 h heat shock and recovery. The nematodes were collected by centrifugation, rinsed once in cold 0.14 M NaCl, twice in Buffer A (20 mM Tris pH 7.4, 20 mM NaCl, 1 mM EDTA, 0.1 mM $MgCl_2$, 10% glycerol), frozen by dripping them as a thick suspension into liquid nitrogen, and stored at −70°C. Nematodes were labelled with ^{35}S as described previously [5].

2.2. *Preparation of extracts*

Frozen nematode pellets were thawed and diluted to approximately 0.1 g of nematodes per ml of buffer A in a 15 ml Corex centrifuge tube. The temperature was maintained at 0–4°C throughout. Phenylmethylsulfonyl fluoride was added to 0.2 mM and the mixture homogenized with 5 to 6 ten second full power bursts from a sonicator fitted with a microtip (Heat Systems Inc.). The solution was cooled on ice between bursts. The homogenate was then centrifuged at 20 000 × *g* for 20 min. The supernatant was carefully removed and filtered

through a syringe fitted with a 0.22 micron filter. For larger scale preparations, nematodes were homogenized in a French Press at 10 000 psi.

Proteins were analyzed using the discontinuous SDS-polyacrylamide gel system of Laemmli [11]. Slab gels consisted of an 18% separating gel with a 4.5% stacking gel. Gels were run at 80–100 V until the Bromophenol blue marker rann off the end. Two-dimensional gel electrophoresis was carried out according to the procedure of O'Farrell [12].

2.3. *Immunological techniques*

Peptides were synthesized using tertiary butyloxycarbonyl solid phase chemistry on an Applied Biosystems 430A Peptide Synthesizer and purified by reverse phase HPLC [13]. Peptides corresponding to amino acids 125–143 of the hsp16-41 gene, 33–50 of the hsp16-1 gene, and 110–145 of the hsp16-2 gene were made. Peptides 16-41 (125–143) and 16-2 (110–145) had a cysteine residue added to the N-terminus to facilitate linkage to maleimide modified KLH or to Sepharose [14]. The 16-1 (33–50) peptide includes a natural cysteine residue at its N-terminus. New Zealand White rabbits were immunized with 0.25 to 0.5 mg of peptide conjugated to keyhole limpet hemocyanin (KLH) in an emulsion of Freund's complete adjuvant and sterile 0.14 M NaCl (3:1 ratio). The animals were bled and boosted at three week intervals using Freund's incomplete adjuvant. Immunoglobulins were precipitated with 45% ammonium sulfate and purified by affinity chromatography on thiopropyl-Sepharose 6B (Pharmacia) containing the appropriate peptide linked via its cysteinyl residue.

Proteins on SDS-polyacrylamide gels were electroblotted to polyvinylidene difluoride (Immobilon-P, Millipore) membranes using a method similar to that for nitrocellulose [15].

2.4. *Column chromatography*

Hydroxylapatite was hydrated with degassed Buffer A, decanted once and packed into a column at 4°C. The filtered extract was loaded onto the column and washed with 1.5 column volumes of buffer A and 1.5 column volumes of Buffer B (10 mM potassium phosphate pH 7.4, 20 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol). It was eluted first with 1.5 column volumes of Buffer B/50 (Buffer B except containing 50 mM potassium phosphate) and then Buffer B/200 (Buffer B containing 200 mM potassium phosphate). Concentrated samples (2 ml) of these eluates were fractionated on a 1.6 × 100 cm S-300 Sephacryl gel filtration column (Pharmacia) packed in Buffer A containing 0.1 M NaCl.

3. RESULTS AND DISCUSSION

Fig. 1 shows that on the Western blot each of the anti-hsp16 peptide antibodies detects primarily a single polypeptide which appears only in extracts from heat shock induced nematodes. Inspection of the autoradiograms indicated that the anti-1/33–50 and anti-2/110–145 antibodies detected an 18 kDa band while the anti-41/125–143 antibody detected a 16 kDa band. In addition, the anti-2/110–145 antibody faintly detected the 16 kDa band while the anti-41/125–143 antibody faintly detected the 18 kDa band. This antibody specificity correlates well with the extent of sequence similarity between the hsp16 proteins. As can be seen in Fig. 2, hsp16-1 and hsp16-2 are highly similar to each other but are only 50% identical to hsp16-41 and hsp16-48 at positions 110–145 and virtually unrelated at positions 33–50. Similarly, hsp 16-41 and hsp16-48 are identical at positions 125–143 but are only 45% identical to hsp16-1 and hsp16-2. These results suggest that the 18 kDa heat shock polypeptide may not be a distinct

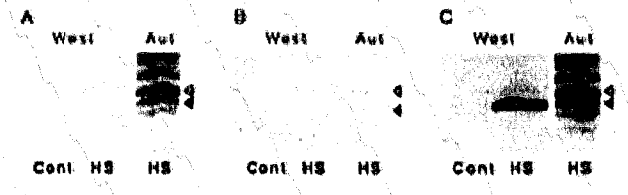

Fig. 1. Western blot analysis of ^{35}S-labelled control and heat shocked nematode extracts. Western blots (West) of control (Cont) and heat shocked (HS) extracts were developed with (A) anti/1-33-50, (B) anti-2/110-145, and (C) anti-41/125-143 primary antibody and horseradish peroxidase goat-anti-rabbit secondary antibody. Autoradiograms (Aut) of the heat shock lane of each blot are aligned vertically to the left of their respective blots. The solid arrowhead marks the 16 kDa polypeptide and the hollow arrowhead marks the 18 kDa polypeptide.

small hsp as previously believed [5,16] but may in fact be encoded by the hsp16-1 and hsp16-2 genes, while the 16 kDa heat shock polypeptide may be encoded by the hsp16-41 and hsp16-48 genes. If this is so, the lower mobility of the 16-1/2 pair relative to the 16-41/48 pair must be due to the existence of an SDS-stable conformation in these proteins, or to postsynthetic modifications, since all four hsps have molecular masses of 16 kDa based on the amino acid sequences derived from their genes [6,7]. Attempts to confirm the identity of the 18 kDa hsp by direct microsequencing of gel-purified proteins [19] were unsuccessful, suggesting that the amino-terminal is blocked.

The heterogeneity of the hsp16 polypeptides and the specificity of the anti-2/110–145 antibody were further examined by two-dimensional gel electrophoresis (Fig. 3). A total of 13 heat-induced polypeptides were seen in the 16–18 kDa region of the gel (Fig. 3B). The anti-2/110–145 antibody reacted strongly with the components of highest apparent molecular weight, and weakly with the others (Fig. 3D). None of these polypeptides were detectable in non-heat-shocked animals (Fig. 3A,C). The existence of only four known

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          33              1/33-50
          |
HSP16-1   CRGSPSESSEIVNNDQKF
HSP16-2   CRGIPSESSEIVNNDQKF
HSP16-41  sfnfsdnigeIVNdesKF
HSP16-48  sfnfsdnigeIvndesKF

         110              2/110-145
          |
HSP16-1   NLSEDGKLSIEAPKKEAiQGRSIPIQQApVEqKtsE
HSP16-2   NLSEDGKLSIEAPKKEAVQGRSIPIQQAIVEEKSAE
HSP16-41  aiSneGKLqIEAPKKtnss-RSIPInfvakh
HSP16-48  aiSneGKLqIEAPKKtnss-RSIPInfvakh

         125              41/125-143
          |
HSP16-1   APKKeaiqgRSIPIqqapveqktse
HSP16-2   APKKeavqgRSIPIqqaiveeksae
HSP16-41  APKKTNSS-RSIPINFVAKH
HSP16-48  APKKTNSS-RSIPINFVAKH
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Fig. 2. Amino acid sequences of the hsp16 regions which correspond to the synthetic peptides used in this work. Peptides were synthesized for the protein products of the genes named in bold type. Upper case letters represent, in the single amino acid code, those amino acids identical to the peptide. Lower case letters represent non-identity to the peptide. Amino acid sequences are from [6,7].

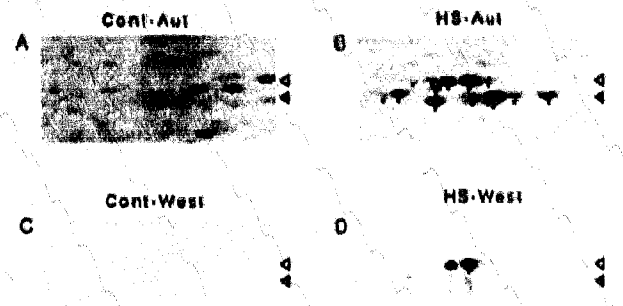

Fig. 3. Autoradiographic and Western blot analysis of two-dimensional gel electrophoretic separations of control (Cont) and heat shocked (HS) ^{35}S-labelled nematode extracts. (A) and (B) are autoradiograms (aut) of dried gels. (C) and (D) are Western blots developed with anti/2/110–145 primary and goat anti-rabbit-alkaline phosphatase secondary antibodies. The solid dots in (B) are below each spot not found in (A). The solid and hollow arrowheads are as in Fig. 1. The pH gradient of each panel is approximately 6 to 8 from left to right.

hsp16 genes in the *C. elegans* genome [6,7] suggests that some of the protein components may arise from post-translational modifications.

The molecular weight of the native hsp16/18 proteins was investigated by gel exclusion chromatography on Sephacryl S-300 columns. The column profile with molecular weight calibration is shown in Fig. 4A, and the Western blot of selected column fractions in Fig. 4B. It is seen that all of the hsp16/18 detected by this antibody migrated in the region of the column corresponding to a molecular weight range of $4\text{–}5\times10^5$. No hsps were detected in the region corresponding to the monomeric proteins. Thus the hsp16/18 proteins of *C. elegans* exist as high molecular weight aggregates, as do the related 20–30 kDa hsps of *Drosophila* [4] and vertebrate cells [17,18].

The nematode small hsps are approximately 10 kDa smaller than the corresponding *Drosophila* and mammalian proteins, and the sequence similarity among these proteins corresponds to the region of homology with the vertebrate alpha-crystallins [6]. This suggests that this homologous region may correspond to the aggregating domain in these proteins. Indeed, the region of maximum sequence similarity among the four hsp16 sequences of *C. elegans* corresponds to exon 2 of their genes, and this exon constitutes the region homologous to alpha-crystallins and to the *Drosophila* small hsps [6,7]. Exon 1, in contrast, is divergent not only relative to the small hsps of other species, but within the hsp16 family of *C. elegans* as well. It seems likely, therefore, that exon 2 of the hsp16's represents the minimal protein structure capable of aggregating in such a way as to fulfill the functional role of these hsps in the cell.

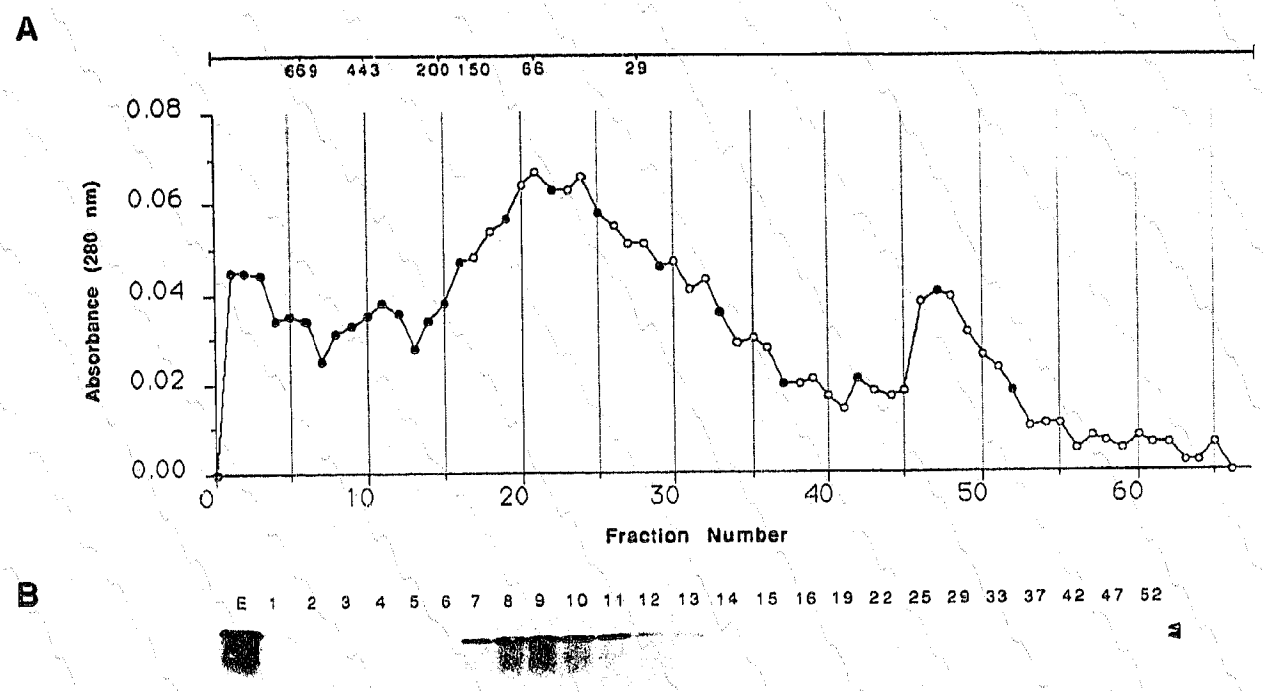

Fig. 4. Gel filtration fractionation of a hydroxylapatite purified heat shocked nematode extract. (A), elution profile of the column showing the absorbance of each 2 ml fraction after the void volume. The elution positions of standard proteins are shown above the profile (kDa). Solid circles, fractions which were analyzed by Western blot (B) as in Fig. 3. 'E', Buffer B/50 hydroxylapatite fraction. The numbers in (B) correspond to the fraction numbers in (A). The solid and hollow arrowheads are as in Fig. 1.

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