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The Expanding Small Heat-Shock Protein Family, and Structure Predictions of the Conserved “ α -Crystallin Domain”

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Abstract. The ever-increasing number of proteins identified as belonging to the family of small heat-shock proteins (shsps) and α -crystallins enables us to reassess the phylogeny of this ubiquitous protein family. While the prokaryotic and fungal representatives are not properly resolved, most of the plant and animal shsps and related proteins are clearly grouped in distinct clades, reflecting a history of repeated gene duplications. The members of the shsp family are characterized by the presence of a conserved homologous “ α -crystallin domain,” which sometimes is present in duplicate. Predictions are made of secondary structure and solvent accessibility of this domain, which together with hydropathy profiles and intron positions support the presence of two similar hydrophobic β -sheet-rich motifs, connected by a hydrophilic α -helical region. Together with an overview of the newly characterized members of the shsp family, these data help to define this family as being involved as stable structural proteins and as molecular chaperones during normal development and induced under pathological and stressful conditions.

Key words: Molecular chaperones — Molecular phylogeny — Molecular evolution — Structural domains — Structure prediction

Introduction

The small heat-shock proteins (shsps) are one of the four most common groups of heat-shock proteins (Lindquist and Craig 1988; Hendrick and Hartl 1993; Morimoto et al. 1994). These low-molecular-weight proteins are evolutionarily related to the vertebrate lens protein α -crystallin, as was first noted by Ingolia and Craig (1982). The shsps are a diverse family of proteins of 15–30 kDa which tend to form large aggregates. Most species have multiple shsp genes, although in yeast and chicken only one gene was detected (Susek and Lindquist 1989; Miron et al. 1991). In plants, the shsp genes are most numerous (Vierling 1991). Apart from two multigene families of cytoplasmic shsps (Raschke et al. 1988), plants have also nucleus-encoded shsps that localize to chloroplasts (Chen and Vierling 1991). Recently, a fourth group of plant shsps was found which are localized in the endomembrane system, most likely in the endoplasmic reticulum (Helm et al. 1993).

α -Crystallin is an abundant eye lens protein in vertebrates. It is usually found as large aggregates, consisting of two types of subunits, α A and α B. (For reviews see Wistow and Piatigorsky 1988; Groenen et al. 1994.) It has a structural function in the lens, warranting proper refractive properties and transparency (Tardieu and Delaye 1988). α B- and to a lesser extent α A-crystallin have been shown to occur also in various tissues outside the lens (Bhat and Nagineni 1989; Kato et al. 1991). Both subunits are encoded by single-copy genes in humans. Functionally, the shsps and α -crystallins share the prop-

erty of being molecular chaperones (Horwitz 1992; Jakob et al. 1993), and both are able to convey thermotolerance (Landry et al. 1989; Klemenzt et al. 1991).

Apart from the shsps and α -crystallins, this family also includes more disparate members, like certain surface antigens in parasitic eukaryotes and bacteria (Nene et al. 1986; Nerland et al. 1988; Verbon et al. 1992). All members of the family are characterized by the presence of a homologous sequence of about 80 residues, which has been dubbed the " α -crystallin domain" and probably forms a distinct structural and functional unit. This domain is preceded by an N-terminal region of variable length, which shows little or no similarity between the various branches of the family. A short and variable sequence, but containing a conserved motif, extends C-terminally from the " α -crystallin domain" (Wistow 1985; de Jong et al. 1988). Unfortunately, no direct information about the three-dimensional structure of any member of the shsp family is available, nor is there any deeper insight into their structural roles and functional activities.

The α -crystallin/small hsp family is not related to any other protein family, although Lee et al. (1993) noticed some minor sequence similarities between the " α -crystallin domain" and two separate regions in the hsp70 family. This might reflect the sharing of some structural features, possibly pertaining to the shared chaperone functioning, rather than an evolutionary relationship.

Recently, a considerable number of sequences of diverse new members of the shsp family have been reported. A comparison of the sequences and the structural and functional properties of all members of the family should contribute to a more detailed view of the divergent evolution of this family. The objective of the present paper is to give an update of the phylogenies presented earlier (Plesofsky-Vig et al. 1992; de Jong et al. 1993), as well as to analyze the structural and functional variation and similarity of the proteins. The latter information might be important for the future unravelling of the structure-function of especially the conserved " α -crystallin domain."

New Members of the Family

A description of the newly characterized members of the shsp family further highlights the variety of features presented by this family, as revealed already earlier (de Jong et al. 1993). The 16-kDa *Escherichia coli* IbpA and IbpB proteins, which are 52.2% identical, were found to be induced in response to expression of several heterologous proteins (Allen et al. 1992). They are tightly associated with inclusion bodies formed during the expression of these foreign proteins. The presence of a high level of certain unfolded heterologous proteins may in fact be responsible for induction of IbpA and IbpB. Also, induction occurred in wild-type *Escherichia* after heat shock. A putative heat-shock promoter is indeed located

upstream from the genes, which are separated by 110 bp on the chromosome.

A *Clostridium acetobutylicum* hsp18 was shown to be induced at the mRNA level by heat shock and the onset of solventogenesis, a metabolic shift in which excreted butyric acid and residual sugar are converted to acetone and butanol shortly before entry into the stationary phase (Sauer and Dürre 1993). The shift to solventogenesis seems to be connected with the heat-shock response, but the underlying molecular mechanisms are still largely unknown. In the myxobacterium *Stigmatella aurantiaca* a 21-kDa protein, SP21, is synthesized during heat shock, fruiting body formation, and stress induced by oxygen limitation (Heidelbach et al. 1993a,b). The protein sedimented with the membrane fraction. As there were no indications of a direct interaction of SP21 with the membranes, this suggests that SP21 forms aggregates or is organized in larger complexes.

In the eukaryotic realm, several new plant sequences were added to the set, including shsps of *Arabidopsis thaliana* (Bartling et al. 1992), maize *Zea mays* (Jorgensen and Nguyen 1994), wheat *Triticum aestivum* (Weng et al. 1991) and rice *Oryza sativa* (Tseng et al. 1992; R. Nishi et al., data base acc. nr. P31673). Most of these sequences were determined at the cDNA level. The gene of the *Pharbitis nil* 17.1-kDa shsp was induced by light treatment and by heat shock, whereas its 18.8-kDa gene was induced only by heat shock. Both are encoded by one open reading frame. In the noncoding region of both genes several heat-shock elements were found (Krishna et al. 1992). The *Chenopodium rubrum* 18.3-kDa shsp belongs to the cytosolic shsp subfamily (class I). A structure with "positive DNA-binding regulatory properties" was predicted that would form a helix-turn-helix region (Knack et al. 1992).

Maximal accumulation of the *Helianthus annuus* shsp mRNA is detected in dry seeds and during embryo mid-maturation stage in the absence of exogenous stress (Almoguera and Jordano 1992). In seedlings, mRNA accumulation to lower levels is found in response to osmotic stress and abscisic acid treatments. The protein has a predicted weight of 17.6 kDa. A 22-kDa shsp from the soybean *Glycine max* was found to be endomembrane localized. It possesses an amino-terminal signal peptide and a carboxyl-terminal sequence characteristic of an endoplasmic reticulum retention signal (Helm et al. 1993). The expression of meiotic prophase repeat proteins in *Lilium* sp. is developmentally induced in absence of stress. A cDNA sequence for one of these proteins was determined, and demonstrated it to be a shsp (Bouchard 1990).

Among the invertebrates, an additional *Schistosoma mansoni* egg antigen sequence, p40-2 (Cao et al. 1993), has 57% amino acid identity to the previously reported p40 antigen (Nene et al. 1986), and likewise contains a duplicate " α -crystallin domain." These stage-specific

[illegible]

Glycine_15	DMKETQ.EAH.VLKAIDFG.L.K.KE..EVKV.QLEDDRVLIQI.SG..ERNVEK.EDK....NDT.WHR..VDRSS.GKPMRRFRPLP.EN..AKVEQV.KACH.ENGVL.TVTVPK...E.EVKK...SDV.KPIEISG...
Glycine_16	DMKETP.EAH.VFAIDFG.L.K.KE..EVKV.QLEDDRVLIQI.SG..ERNVEK.EEK....NDT.WHR..VERSS.GKPMRRFRPLP.EN..AKVEQV.KACH.ENGVL.TVTVPK...E.EVKK...POV.KALEISG...
Chenopodium_18	DMKETP.EAH.VFAIDFG.V.K.KE..EVKV.EVEDGNVLI.SG..QARREK.EEK....NDT.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EAPK...POV.KALINVT...
Triticum_16	DMKETP.EAH.VFAIDFG.V.K.KE..EVKV.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GQPMRRFRPLP.DD..AKVEEV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Zea_172	DMKETP.EAH.VFAIDFG.V.K.KE..EVKV.EVEDGNVLI.SG..QARREK.EEK....NDK.WHR..VERSS.GQPMRRFRPLP.DD..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Oryza_17a	DMKETP.EAH.VFAIDFG.V.K.KE..EVKV.EVEDGNVLI.SG..QARREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Glycine_17b	DMKETP.EAH.VFAIDFG.L.K.KE..EVKV.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Glycine_22a	DMKETP.EAH.VFAIDFG.L.K.KE..EVKV.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Plum_22	DMKETP.EAH.VFAIDFG.L.K.KE..EVKV.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Lilium_6	DMKETP.EAH.VFAIDFG.L.K.KE..EVKV.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Pharbitis_19	DMKETP.EAH.VFAIDFG.V.R.TG..DIKI.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Glycine_61	DMKETP.EAH.VFAIDFG.V.R.TG..DIKI.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Plum_17	DMKETP.EAH.VFAIDFG.V.R.TG..DIKI.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Arabidopsis_17b	DMKETP.EAH.VFAIDFG.V.R.TG..DIKI.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Zea_18a	DMKETP.EAH.VFAIDFG.V.R.TG..DIKI.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Zea_18b	DMKETP.EAH.VFAIDFG.V.R.TG..DIKI.EVEDGNVLI.SG..ERSREK.EEK....NDK.WHR..VERSS.GKPMRRFRPLP.EN..AKVDQV.KAGL.ENGVL.TVTVPK...A.EVKK...PEV.KALEISG...
Consensus	-----D-PG-- --S-KV -V-D-VI-I -G-----D-R-----V-A-I--GVLT--PK-----I-I-----

Fig. 1. Alignment of the homologous C-terminal regions of the shps and related proteins. Sequences are designated by *genus name* and, for the small hsp, the *numbers* as used in the databases. These numbers can be different from those used in the literature. *aA* and *aB* = α A- and α B-crystallin sequences, respectively. *Schistosoma-N1*, -N2, -C1, and -C2 = N- and C-terminal copies, respectively, of the duplicated α -crystallin domains in the egg antigen p40 and p40-2. *Mycobac lep* and *Mycobac tub* = *Mycobacterium leprae* and *M. tuberculosis*. *Cl* = chloroplast-localized hsp. The positions corresponding to the introns in the genes for *Caenorhabditis elegans* hsp (1, 2), *Halocynthia roretzi* HR-29 (3, 4), mammalian hsp27 (5, 6), and α A- and α B-crystallins (1, 7) are indicated with arrows above the alignment. All introns fall between codons (phase 0), apart from #4, which is between the second and third nucleotides in the codon for residue 70 (phase 2). "Consensus" indicates residues that are present in 50% or more of the entries at a given position. To the alignment of de Jong et al. (1993) the following sequences are added: *Gallus gallus* α A-crystallin (database accession number P02504, residues 63–173), *G. gallus* α B-crystallin (Q05713, 66–174), *Homo sapiens* p20 (Kato et al. 1994, 66–160), *Rattus norvegicus* p20 (Kato et al. 1994, 66–162), *R. norvegicus* hsp27 (R.R. Gilmont and M.J. Welsh, M86389, 91–206), *Nippostrongylus brasiliensis* hsp20 (S33416, 57–172), *Cae norhabditis elegans* hsp12.3 (L15188, translation of residues 36,675–36,752, 37,383–37,496,

37,904–38,044), *Acanthocheilonema viteae* AV25 (S29691, 62–179), *Onchocerca volvulus* OV25-1 (S29692, 61–173), *O. volvulus* OV25-2 (S29693, 49–165), *Halocynthia roretzi* HR-29 (JX0258, 147–252), *Schistosoma mansoni* p40-2 (M96866, 128–253 and 254–342), *Escherichia coli* lbpA (A45245, 39–137), *E. coli* lbpB (B45245, 37–142), *Clostridium acetobutylicum* hsp18 (S25534, 48–151), *Stigmatella aurantiaca* SP21 (M94510, 57–188), *Arabidopsis thaliana* hsp17.6 (P29830, 49–155), *Zea mays* hsp17.2 (X65725, 48–152), *Triticum aestivum* hsp26.6 (Q00445, 134–236), *Oryza sativa* hsp17a (P27777, 46–150), *O. sativa* hsp17b (P31673, 50–154), *Pharbitis nil* hsp17 (Q01544, 48–155), *P. nil* hsp19 (Q01545, 59–167), *Chenopodium rubrum* hsp18 (Q05832, 58–161), *Helianthus annuus* hsp18 (P30693, 48–153), *Glycine max* hsp22 (P30236, 70–192), *Lilium* sp. EMPR6 (A61054, 43–146). The *Homo sapiens* α A-crystallin sequence underwent a minor correction at positions 114–118 of the alignment according to Takemoto and Emmons (1991) and Caspers et al. (1994). The *Mus* hsp27 has been corrected as described by Gaestel et al. (1993) and Merck et al. (1993b). To avoid undue lengthening of the alignment, two unique sequences are left out (marked by open arrowheads): a 64-residue insert after position 47 in *Neurospora* hsp30 (A), and a 15-residue insert after position 104 in *Lilium* EMPR6 (B).

antigens are soluble calcium-binding proteins (Moser et al. 1992). Expression of the gene for the shsp of the gastrointestinal nematode *Nippostrongylus brasiliensis* is developmentally regulated, but not upregulated by heat shock or other stress conditions (Tweedie et al. 1993). Another new nematode shsp sequence was found on chromosome III of *Caenorhabditis elegans* (Wilson et al. 1994). Also two sequences of *Onchocerca volvulus* and one of *Acanthocheilonema viteae* are now available (W. Hoefle, acc. nrs. S29691–3).

A new shoot of the family is represented by the 29-kDa protein HR-29 of the ascidian *Halocynthia roretzi* (Takagi et al. 1993). The protein is abundant in body wall muscle and localizes close to the plasma membrane. In the physiological condition it forms oligomers, observed by electron microscopy as globular aggregates with a diameter of 16.6 nm, as is also the approximate size for α -crystallin and shsps.

Finally, an additional shsp was recently reported in mammals. Kato et al. (1994) found high levels of a 20-kDa protein, p20, in rat soleus muscle, heart, and diaphragm. These tissues also contain high levels of α B-crystallin and hsp27. The p20 protein is present at lower levels in other tissues. It occurs both as high-molecular-weight aggregates and in dissociated forms. Upon heating at 45°C of rat diaphragm in vitro, p20 was redistributed from the cytoplasm to the insoluble fraction, and dissociation of the aggregated p20 to the small form was enhanced. The primary structure of rat p20 and its human homologue were determined at the amino acid level.

Sequence Comparisons and Gene Structure

To ensure that all available shsps and related sequences were included in the present paper, a thorough database search was performed. To that end a previous alignment of 57 sequences of “ α -crystallin domains” (de Jong et al. 1993) was used to create a profile (Gribskov et al. 1990) with which the NBRF, SwissProt, and EMBL databases were searched to identify new sequences with similarities to the shsps. These were included in the expanded alignment. As for the extensive set of available α -crystallin sequences, only those for chicken α A- and α B-crystallin (de Jong et al. 1984; Sawada et al. 1992) were added to the previous alignment. Initial alignments of the expanded set of 85 entries were made with a multiple alignment program, followed by manual improvements, as described earlier (de Jong et al. 1993). Because a clear similarity among all members of the family is restricted to the C-terminal parts of the proteins, only this region could be aligned satisfactorily, as shown in Fig. 1. In the expanded shsp family more gaps needed to be introduced in the alignment of this conserved region. The region presented in Fig. 1 corresponds with the putative C-terminal structural domain and extending tail of the α -crystallins and shsps as proposed by Wistow

(1985). A considerable number of residues is highly conserved throughout the family (indicated as “consensus” on the bottom line in Fig. 1), and the conservative nature of many other positions is conspicuous. The demarcation between domain and tail is around position 110.

The positions of the conserved “ α -crystallin domain” in the various typical representatives of the shsp family are schematically indicated in Fig. 2. Both *Schistosoma* egg antigen sequences contain duplicate C-terminal domains. The C-terminal domain of the *Neurospora* shsp is interrupted by an insertion of 64 amino acids (after position 47 in the alignment). This figure also reveals the length variation in the family. The length of the N-terminal regions, preceding the α -crystallin-like domains, varies from only 25 residues in the *Caenorhabditis* 123 gene that was found in the course of the chromosome III sequencing project (Wilson et al. 1994) to 148 residues in the *Halocynthia roretzi* 29-kDa body-wall protein. Although no sequence similarity can be detected between the N-terminal regions of all members of the family, there are still some minor similarities between the N-termini of the α -crystallins, higher vertebrate shsps, and *Drosophila* shsps, as well as between plant class I shsps and vertebrate shsps (de Jong et al. 1988). It must be concluded that the rate of evolution has been higher in the N-termini than in the C-terminal regions.

In plants, yeast, and most invertebrates, the shsp genes are encoded by intronless genes. Four shsp genes of *Caenorhabditis* contain only one intron, coinciding precisely with the first intron of the α -crystallin genes (Figs. 1 and 2). The aforementioned *Caenorhabditis* 123 gene contains two introns, of which the second coincides approximately with the second intron of the α -crystallin genes. The two introns of mammalian hsp27 are located at different positions. The *Halocynthia* body-wall protein gene contains three introns, of which the second is located three nucleotides downstream from intron #1 in *Caenorhabditis* shsps and in α -crystallins, thus directly preceding the C-terminal domain. The other two introns are located at different positions than those in other genes of the family and are phase 2, rather than phase 0 as are all other ones (Takagi et al. 1993). Although introns #1 and 3 in Fig. 2 demarcate the boundary between the putative N- and C-terminal domains, there is little evidence for the assumption that exon shuffling has played a role in the evolution of this protein family (Patthy 1994).

Phylogeny

Residues 1–104 of the alignment in Fig. 1, encompassing the “ α -crystallin domain,” were used to construct a phylogenetic tree, using the neighbor-joining program (Saitou and Nei 1987) from the PHYLIP package (Felsenstein 1993). The “ α -crystallin domain” was considered

Fig. 2. Location of the homologous domains and intron positions in representative members of the shsp family. The homologous C-terminal “ α -crystallin domains” are indicated by solid bars. N-terminal domains and C-terminal extensions are indicated by lines, corresponding in length to the number of residues. Intron positions in the corresponding genes are marked by arrowheads. Whether introns are present in the *Schistosoma* p40 genes is not known. Intron positions are more precisely indicated in Fig. 1 and its legends. The position of the insertion in *Neurospora* hsp30 is indicated by the open triangle. Names of the sequences are as in Fig. 1.

not to extend beyond residue 104, because after this position a 15-residue insert in the *Lilium* sequence breaks up the alignment. Since the structures of all members of the family are not known at the DNA level, the amino acid sequences rather than the nucleotide sequences were used in tree construction. The resulting tree is shown in Fig. 3, where bootstrap values of 75% and higher are indicated. Because of the large number of sequences, the tree is depicted in three parts, comprising prokaryotes and lower eukaryotes (Fig. 3a), and the higher plant and animal subtrees (Fig. 3b and c, respectively). The sequences at the deepest branches of the tree, the prokaryote and fungal proteins, are very divergent and not reliably resolved (Fig. 3a). Only the two *Escherichia* Ibp proteins group significantly together. The *Mycobacterium* antigens do not form a sister group, and neither do the *Neurospora* and *Saccharomyces* shsps. However, these proteins need not be orthologues; it is likely that only a small subset of the existing shsp variation has been discovered yet. The present findings indicate that reconstruction of the earliest divergent evolution of the shsps will be problematic.

The angiosperm sequences group together, although at a low bootstrap value (Fig. 3b). A first divergence occurs between the chloroplast sequences and the cytoplasmic shsps. The latter divide highly significantly into the class I and class II sequences (Vierling 1991), which

must have emerged before the divergence of monocots and dicots. Repeated duplications have occurred, especially of class I genes. The *Lilium* meiotic prophase repeat protein appears to be most closely related to the class II proteins. The endomembrane-localized *Pisum* and *Glycine* shsps represent an early offshoot of the class I proteins, as was also noted by Helm et al. (1993).

A monophyletic origin of the animal sequences is hardly supported (Fig. 3c). The duplicated N- and C-domains within the two *Schistosoma* egg antigens are very divergent. The two antigens are apparently the result of a more recent gene duplication, long after the emergence of the first antigen with two “ α -crystallin domains.”

All nematode sequences group together, as do all *Drosophila* sequences, albeit poorly supported. It was noted earlier that the *Caenorhabditis* shsp genes duplicated at an early stage, followed by a more recent, further duplication (Candido et al. 1989). The new *Caenorhabditis* sequence (Wilson et al. 1994) appears to represent a different lineage, as it is more closely related to the *Onchocerca* and *Acanthocheilonema* sequences. It was tabulated as most similar to α B-crystallin by Wilson et al. (1994), but the present phylogenetic analysis reveals no special relationship. The two *Onchocerca* shsps appear to have arisen from a very recent gene duplication.

As was noted before, the *Xenopus* shsps are not closely related to other vertebrate shsps, and may in fact be paralogues of these (de Jong et al. 1988). Interestingly, the body-wall protein of the ascidian *Halocynthia* appears to be most closely related to the *Xenopus* shsps. The other vertebrate shsps form a clade with the α A- and α B-crystallins. In this clade, the recently discovered rat and human p20 proteins form a sister group to the monophyletic α A- and α B-crystallins. They are not closer to α B-crystallins, as Kato et al. (1994) assumed. Three gene duplications thus have led to the expression of four paralogous genes in higher vertebrates—hsp27, p20, and α A- and α B-crystallin. These duplications preceded the earliest vertebrate radiation, since α A- and α B-crystallins were already present before the dogfish *Squalus* diverged from the other vertebrates. Additional duplications must have occurred in the hsp27 lineage. The human hsp27 gene family consists of four members (Hickey et al. 1986), although only a single sequence has been reported. Cooper and Uoshima (1994) found multiple hsp27 transcripts in murine osteoblasts, which may be encoded by separate genes. Also a mouse hsp27 pseudogene has been characterized (Fröhli et al. 1993).

Secondary Structure and Surface Residues of the “ α -Crystallin Domain”

Insight into the properties of the shsp family is seriously hampered by the lack of structural information. Using advanced prediction methods on the accumulated se-

Secondary structure predictions of the α -crystallin domain were done for subsets of the alignment, using the program PHD (Rost and Sander 1993; Rost et al. 1994) via the PredictProtein e-mail server at EMBL, Heidelberg. Solvent accessibility prediction was according to Rost and Sander (1994), via the same e-mail server. The results are shown in Fig. 4. For all subsets, the secondary structure predictions start with a short α -helix, followed by three β -sheet stretches, in the first half of the homologous domain. This $\alpha\beta\beta\beta$ motif is repeated in the second half of the domain, although in plant shsps there are two initial α -helices and an α -helix stretch is predicted in-

The residues of the last β -sheet stretch of both motifs, as well as the residues of the first β -sheet stretch of the first motif, are predicted to be buried, while the α -helix between the motifs is predicted to be in an exposed region. Surface residue predictions of the other parts of the sequence are more equivocal. The location of introns, the large insertion in *Neurospora* hsp30, and the extensive presence of gaps in the region 43–71 (cf. Fig. 1) also make it likely that this is a more flexible and exposed region connecting the two more rigid β -sheet-rich motifs.

Fig. 4. Secondary structure prediction (PHD) and solvent accessibility prediction (Acc) by the methods of Rost and Sander (1993, 1994) for the C-terminal 'α-crystallin domain' of subsets of the shsp family (animal, animal sequences; plant, plant sequences; overall, bacterial and fungal sequences plus *Chlamydomonas* hsp22, *Petunia* hsp21, *Zea* hsp18a, *Helianthus*, *Schistosoma* p40 N-terminal domain, *Caenorhabditis* hsp12, *Drosophila* hsp27, *Xenopus* hsp30c, *Bos* αB-crystallin, and *Halocynthia* HR-29). Amino acid position numbers are as in Fig. 1. A, predicted α-helix; B, predicted β-sheet; e, predicted exposed residue; b, predicted buried residue; -, no prediction made. The positions of introns in the conserved domains are indicated by arrowheads as in Figs. 1 and 2.

This is also the position of the helix-turn-helix with DNA-binding properties suggested to be present in *Chenopodium* shsp (Knack et al. 1992). The possible presence of two similar structural motifs in the C-terminal domain of α-crystallin was first proposed by Wistow (1985) and appears to be corroborated by the present data.

The composite hydrophobicity profile (Fig. 5) confirms the two-motif structure of the 'α-crystallin domain.' Peaks of hydrophobicity concur with the first β-sheet stretches in both motifs, whereas also the other β-sheet stretches are located in the more hydrophobic regions, in agreement with the predicted buried position of these stretches. The exposed α-helix that connects the two motifs locates with the strongly hydrophilic region around position 50. The C-terminal arm, beyond position 110, also has a more hydrophilic character. In α-crystallins, the terminal eight to ten residues indeed form very flexible extensions, as revealed by two-dimensional NMR spectroscopy (Carver et al. 1992).

Evolution of Function

It is logical to assume that the conserved C-terminal domain would be responsible for the common structural and functional properties of the α-crystallin/shsp family. (For review of the latter, see Arrigo and Landry 1994.) Deletion of the last 42 amino acids of this domain in *Drosophila* hsp27 indeed abolishes its capacity to protect cells against heat stress (Mehlen et al. 1993). However, in α-crystallins and mouse hsp25 this separate domain with its C-terminal extension has no chaperone activity in vitro (Merck et al. 1993b). It thus seems likely that also the N-terminal domain is required for proper functioning, and may moderate more specific functions for various subgroups of shsps. Considering the multitude of

functions among the members of the shsp family, one might wonder what the primordial function of the common ancestor was. The fact that also in bacteria, like *Escherichia* and *Clostridium*, shsps are now found to be induced by heat shock, suggests that these proteins, too, function in chaperoning other proteins under conditions of stress. The *Escherichia* Ibp proteins also appear to be induced by a high level of unfolded proteins (Allen et al. 1992), which further strengthens the notion that chaperoning is the primordial function of the family, rather than a secondarily developed feature.

The abundant expression of shsps as surface antigens in mycobacteria and in *Schistosoma* must then be one of the various derived functions in the family. For the SP21 protein of the myxobacterium *Stigmatella* it is speculated that it might help pack mRNAs necessary for the early steps of germination or might protect mRNAs of house-keeping genes during periods of development during which other mRNAs are degraded (Heidelbach et al. 1993b). A tight association during heat shock between shsps and a specific subset of mRNAs has earlier been reported for tomato cell cultures (Nover et al. 1989).

Developmental regulation is also well documented in many other members of the family, notably in animal shsps and αB-crystallins (de Jong et al. 1993; Gernold et al. 1993; Marin et al. 1993). Expression of the gene for the shsp of the gastrointestinal nematode *Nippostrongylus brasiliensis* is developmentally regulated (Tweedie et al. 1993). mRNA peak expression is concomitant with the onset of immune damage in the host, but expression is regulated independently of stress stimuli, apparently according to a strict developmental program. In plants, like for *Helianthus* (Almoguera and Jordano 1992) and alfalfa (Györgyey et al. 1991), the pronounced expression of shsp mRNA occurs during embryo midmaturation. Shsp mRNAs are stored for long periods of time in dry seeds of sorghum (Howarth 1990), wheat (Helm and

Fig. 5. Composite hydrophobicity profiles of all "α-crystallin domains" of the shsp family, using the hydrophobicity scale of Sweet and Eisenberg (1983), as produced by the program CAMELEON (version 3.0, Oxford Molecular Ltd, Oxford, UK). Amino acid position numbers

are as in Fig. 1. Hydrophobicity increases toward the top of the figure; hydrophilicity toward the bottom. Predicted β-sheets (see Fig. 4) are indicated by bars.

Abernethy 1990), and pea (Vierling and Sun 1987). All this supports the notion that developmental expression of shsps is quite universal (see Linquist and Craig 1988). The shsps are apparently involved in cytomorphological rearrangements, which may relate to their influence on actin polymerization (Miron et al. 1991) and intermediate filament assembly (Nicholl and Quinlan 1994).

As pointed out by Bouchard (1990), we find an additional non-stress-related function in meiotic cells of three eukaryotic kingdoms. *Lilium* meiotic prophase repeat protein expression is developmentally induced (Bouchard 1990). Two of the four *Drosophila* shsps are expressed in the egg chamber of the ovary during the meiotic period of oogenesis (Zimmerman et al. 1983). The controlling regions for the heat shock and the ovarian induction are different (Hoffman et al. 1987). The *Saccharomyces* shsp is induced during sporulation and meiosis (Kurtz et al. 1986).

Gene duplication is a general mechanism to acquire more functions. For example, in *Pharbitis nil*, one of the shsp genes is induced by heat shock only, the other by light as well (Krishna et al. 1992). The expression of smaller hsp27 transcripts in murine osteoblasts, which may be encoded by separate genes, was facilitated by estrogen treatment prior to heat shock, whereas expression of the normal, longer, hsp27 was induced by heat shock alone (Cooper and Uoshima 1994). Gene duplication must also be the mechanism which enabled an ancestral α-crystallin gene to be recruited for its function as a lens structural protein. Another structural function may be found for the HR-29 protein of *Halocynthia roretzi*, which is localized in body-wall muscle (Shirakata et al. 1986). It may be a component of myofibrils and may act

as a stabilizing protein like αB-crystallin does in skeletal muscle (Atomi et al. 1991).

This brief and necessarily fragmentary account makes it clear that gene duplications and divergent evolution have produced a broad array of structural and functional properties in the shsp family, using the common "α-crystallin domain" as an essential building block. The major challenge for the years to come is to elucidate the tertiary and quaternary structure of members of this family and relate these to their various intriguing functions.

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