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A genomewide analysis of genes for the heat shock protein 70 chaperone system in the ascidian *Ciona intestinalis*

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Abstract Molecular chaperones play crucial roles in various aspects of the biogenesis and maintenance of proteins in the cell. The heat shock protein 70 (HSP70) chaperone system, in which HSP70 proteins act as chaperones, is one of the major molecular chaperone systems conserved among a variety of organisms. To shed light on the evolutionary history of the constituents of the chordate HSP70 chaperone system and to identify all of the components of the HSP70 chaperone system in ascidians, we carried out a comprehensive survey for HSP70s and their cochaperones in the genome of *Ciona intestinalis*. We characterized all members of the *Ciona* HSP70 superfamily, J-proteins, BAG family, and some other types of cochaperones. The *Ciona* genome contains 8 members of the HSP70 superfamily, all of which have human and protostome counterparts. Members of the STCH subfamily of the HSP70 family and members of the HSPA14 subfamily of the HSP110 family are conserved between humans and protostomes but were not found in *Ciona*. The *Ciona* genome encodes 36 J-proteins, 32 of which belong to groups conserved in humans and protostomes. Three proteins seem to be unique to *Ciona*. J-proteins of the RBJ group are conserved between humans and *Ciona* but were not found in protostomes, whereas J-proteins of the DNAJC14, ZCSL3, FLJ13236, and C21orf55 groups are conserved between humans and protostomes but were not found in *Ciona*. J-proteins of the sacsin group seem to be specific to vertebrates. There is also a J-like protein without a conserved HPD tripeptide motif in the *Ciona* genome. The *Ciona* genome encodes 3 types of BAG family proteins, all of which have human and protostome counterparts (BAG1, BAG3, and BAT3). BAG2 group is conserved between humans and protostomes but was not found in *Ciona*, and BAG4 and BAG5 groups seem to be specific to vertebrates. Members for SIL1, UBQLN, UBADC1, TIMM44, GRPEL, and Magmas groups, which are conserved between humans and protostomes, were also found in *Ciona*. No *Ciona* member was retrieved for HSPBP1 group, which is conserved between humans and protostomes. For several groups of the HSP70 superfamily, J-proteins, and other types of cochaperones, multiple members in humans are represented by a single counterpart in *Ciona*. These results show that genes of the HSP70 chaperone system can be distinguished into groups that are shared by vertebrates, *Ciona*, and protostomes, ones shared by vertebrates and protostomes, ones shared by vertebrates and *Ciona*, and ones specific to vertebrates, *Ciona*, or protostomes. These results also demonstrate that the components of the HSP70 chaperone system in *Ciona* are similar to but simpler than those in humans and suggest that changes of the genome in the lineage leading to humans after the separation from that leading to *Ciona* increased the number and diversity of members of the HSP70 chaperone system. Changes of the genome in the lineage leading to *Ciona* also seem to have made the HSP70 chaperone system in this species slightly simpler than that in the common ancestor of humans and *Ciona*.

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INTRODUCTION

Molecular chaperones play crucial roles in various aspects of the biogenesis and maintenance of proteins in the cell (Frydman 2001; Soti et al 2005). They are responsible for the folding of nascent proteins in the cytosol, translocation of proteins into organelles, assembly and disassembly of protein complexes, control of the biological activity of regulatory proteins, renaturation of denatured proteins after stress, and degradation of misfolded or harmful proteins (Hartl and Hayer-Hartl 2002; Craig et al 2003). The heat shock protein 70 (HSP70) chaperone system, in which HSP70 family proteins act as chaperones, is one of the major molecular chaperone systems conserved among a variety of organisms (Bukau and Horwich 1998). In the process of the folding of nascent proteins, for example, HSP70s bind to and release hydrophobic regions of substrate proteins in an ATP-dependent manner. HSP70s in the adenosine 5′-diphosphate (ADP)-bound state bind to the substrate proteins stably, whereas HSP70s in the adenosine triphosphate (ATP)-bound state release them rapidly. The folding of the substrate proteins is thought to be attained through the repetition of this cycle (Fan et al 2003; Mayer and Bukau 2005).

The activity of HSP70s is regulated by many types of cochaperones (Caplan 2003; Mayer and Bukau 2005). For example, members of J-domain–containing protein (J-protein) family stimulate ATP hydrolysis by HSP70s (Wall et al 1994; Tsai and Douglas 1996). BAG-1, a member of the BAG domain–containing protein family (BAG family), acts as a nucleotide exchange factor for HSP70s and accelerates ADP release from HSP70s (Alberti et al 2003). The actions of these cochaperones also affect the substrate specificity of HSP70s and link the HSP70 chaperone system with other chaperone systems and/or other biological systems in the cell, such as the protein degradation pathway (Caplan 2003).

Generally, there are multiple HSP70s and J-proteins in the genomes of eukaryotes, and the number is larger in higher than in lower eukaryotes. For example, the yeast genome contains 14 and 22 genes encoding HSP70s and J-proteins, respectively, whereas the human genome contains 21 and 50 genes, respectively. Although the evolution of HSP70s has been studied extensively (Gupta and Golding 1993; Boorstein et al 1994; Rensing and Maier 1994; Feder and Krebs 1998; Karlin and Brocchieri 1998; Easton et al 2000; Bettencourt and Feder 2001; Nikolaidis and Nei 2004), the details of the changes in the repertoire of HSP70s during the early phases of chordate evolution are unknown. Furthermore, little is known about the evolution of the J-protein family in chordates.

Because the molecular chaperones were originally found as stress-inducible factors, much attention was initially given to their roles in stress responses of the cell. Subsequently, their roles under normal conditions, such as the quality control of proteins in the cell, were investigated intensively. More recently, several studies have focused on the roles of the molecular chaperones in animal development. With regard to the HSP70 chaperone system, for example, it has been reported that some HSP70s are required for spermatogenesis in mouse embryos (Christians et al 2003). DnaJB6, one of the J-proteins, is required for placenta formation in mouse embryos (Hunter et al 1999). *lethal(2) tumorous imaginal discs* is a J-protein-encoding gene required for the differentiation of imaginal disc cells in *Drosophila* (Kurzik-Dumke et al 1995). The results of these studies suggest that the HSP70 chaperone system is involved in various aspects of animal development, the details of which remain to be clarified.

Ascidians are primitive chordates that belong to the subphylum Urochordata of the phylum Chordata. The genome and a total of more than 680 000 expressed sequence tags (ESTs) of *Ciona intestinalis* have been sequenced and it has been shown that the 159-Mb genome contains 15 852 genes that comprise a basic set of chordate-type genes with fewer gene duplications than are observed for vertebrate genes (Dehal et al 2002; Satou et al 2002a, 2002b, 2005). Through rapid and well-described embryogenesis, ascidians form tadpole-like larvae, which are much simpler than but similar to vertebrate embryos. These features make *C intestinalis* one of the excellent model animals to explore the evolutionary trails of vertebrate genes and to investigate the roles of genes in development (Corbo et al 2001; Satoh, 2003; Satoh et al 2003).

In this study, we carried out a comprehensive survey for HSP70s and their cochaperones in the genome of *C intestinalis* with the following 2 aims. One was to shed light on the evolutionary history of the HSP70 chaperone system in chordates by comparing the repertoires of components of the HSP70 chaperone system in *C intestinalis* with those in other animals. The other aim was to identify all of the components of the HSP70 chaperone system in *C intestinalis* to set up a starting point for studies on the roles of the HSP70 chaperone system in ascidian development. We characterized all of the members of HSP70 superfamily, J-proteins, BAG family, and some other types of cochaperones (SIL1, UBQLN, UBADC1, TIMM44, GRPEL, and Magmas) in this ascidian species.

MATERIALS AND METHODS

Searching for *C intestinalis* genes

Search for *C intestinalis* genes was carried out as described in Satou et al (2003). The genome database (http://genome.jgi-psf.org/ciona4/) and cDNA/EST database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) of *C intestinalis* were

screened with all members of HSP70 superfamily, J-protein family, BAG family, SIL1, HSPBP1, UBQLN, UBADC1, TIMM44, GRPEL1/2, and Magmas groups in humans and *Drosophila* as queries using the TBLASTN program with default parameters. Sequences of candidate genes were selected from cDNA sequences (eg, citb001h23), sequences of predicted gene models in the assembled genome sequences (eg, ci0100123456) or EST sequences (eg, cilv003o45EST), giving priority to cDNA sequences. When the predicted gene model seemed to lack part(s) of the gene but the EST(s) covered the region that the gene model lacked, assembled sequence of the gene model and the EST(s) was used for analyses (eg, ciad067d89EST_ci0100123456). All *Ciona* sequences used for analyses are listed in supplementary Table S1.[1] The sequences from other organisms used for analyses are designated by the accession number registered in public databases, abbreviation of the species (HS for human, MM for mouse, CI for *C intestinalis*, DM for *Drosophila melanogaster*, AG for *Anopheles gambiae*, CE for *Caenorhabditis elegans*, SC for *Saccharomyces cerevisiae*, and SP for *Schizosacchromyces pombe*), and gene name. For example, human DNAJA2 (accession number NP_005871) is represented as ''NP_005871_HS_DNAJA2.'' Domains and motifs in the deduced proteins were searched with the SMART (http://smart.embl-heidelberg.de/) and Pfam (http://www.sanger.ac.uk/Software/Pfam/search.shtml), and were summarized in supplementary Tables S2–S5.

Molecular phylogenetic analysis

Molecular phylogenetic analysis was carried out as described in Satou et al (2003). The sequences were aligned using the ClustalX program with default parameters (Thompson et al 1997). As for the tree in supplementary Figure S3, the J-domain sequences were used for the alignment. As for other trees, the full-length sequences were used. The problematic regions that would disturb the alignment (eg, long flanking sequence that is not conserved among most members of a given group) were removed from the sequences used for the alignment before the alignment was performed. The alignment was refined and all gaps were removed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The phylogenetic trees were constructed based on the neighbor-joining method with 1000 bootstrap replicates using MEGA2 (Saitou and Nei 1987; Kumar et al 2001).

Best-hit analysis

In addition to molecular phylogenetic analysis, the orthology of *Ciona* and human proteins was tested by a method that we call best-hit analysis, as described in Satou et al (2003). The given *Ciona* proteins were compared with the human proteome by the BLASTP program using the NCBI web site. The best-hit human protein obtained by this comparison was then used for the TBLASTN search against the *Ciona* genome. In the case that the best-hit sequence of the TBLASTN search coincided with the genomic region encoding the initial *Ciona* protein, the relationship between the 2 proteins suggests their orthology and was called the ''bidirectional best-hit relationship.'' In the other cases, the relationship between the 2 proteins was called the ''unidirectional best-hit relationship.''

[1]Supplementary tables and figures available online at: http://dx.doi.org/10.1379/CSC-137R.s1.

RESULTS

HSP70 superfamily

The HSP70 superfamily consists of the HSP70 family, which contains prototypical HSP70 proteins (Gupta and Golding 1993; Boorstein et al 1994; Rensing and Maier 1994; Karlin and Brocchieri 1998), and the HSP110 family, which is composed of larger, distantly related Hsp110 and homologous proteins (Easton et al 2000). Most members of the HSP70 family in eukaryotes belong to the DnaK subfamily, whereas the remaining, divergent members belong to the STCH subfamily. Members of the DnaK subfamily are classified further into distinct clusters that correspond to their intracellular localization (the cytoplasm, endoplasmic reticulum [ER], mitochondria, and chloroplasts) (Boorstein et al 1994; Lin et al 2001). Similarly to the HSP70 family, the HSP110 family consists of cytoplasmic members and ER-resident members (Easton et al 2000; Nikolaidis and Nei 2004). Cytoplasmic members of the HSP110 family are subdivided into the HSP110/SSE subfamily and HSPA14 subfamily. The ER-resident group of the HSP110 family is called the GRP170 subfamily. Human HSPA12A and HSPA12B form a group (HSPA12 family) that belongs to the HSP70 superfamily but is distinct from the HSP70 family and HSP110 family (Han et al 2003).

In the public databases, 21 human genes encoding HSP70 superfamily–related proteins are registered (HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA4, HSPA4L, HSPA5–8, HSPA9B, HSPA12A, HSPA12B, HSPA14, HSPH1, HYOU1, STCH, LOC401308, LOC442509, LOC402461 and LOC440490). LOC442509, LOC402461, and LOC440490 were excluded from the present analysis, because proteins encoded by them are short and correspond to a limited part of typical HSP70 superfamily proteins. The *Drosophila* genome contains 10 genes for HSP70 superfamily proteins. In the present survey, we identified 8 *Ciona* genes for the HSP70 superfamily proteins (Table 1; supplementary Table S2). The phylogenetic analysis

Table 1 Genes for HSP70 superfamily proteins in the *C intestinalis* genome

Family	Subfamily	Group	Subgroup	Gene name	The best gene model[a]	Scaffold[b]	cDNA cluster[c]	The best-hit protein[d]	Best-hit analysis[e]
HSP70 family	DnaK subfamily	Cytoplasmic group	HSPA1 subgroup HSPA6/7 subgroup	Ci-HSPA1/6/7-like (Cihsp70)	ci0100133183	644	CLSTR33198r1	NP_005336_HS_HSPA1A	<–>
			HSPA2/8 subgroup	Ci-HSPA2/8	ci0100149356	302	CLSTR00331r1	NP_006588_HS_HSPA8 isoform 1	<–>
		ER-resident group		Ci-HSPA5a	ci0100136117	21	CLSTR04514r1	NP_005338_HS_HSPA5	<–>
				Ci-HSPA5b	ci0100138883	21	Not available	NP_005338_HS_HSPA5	–>
		Mitochondrial-type group		Ci-HSPA9B	ci0100154172	2	CLSTR04253r1	NP_004125_HS_HSPA9B	<–>
	STCH subfamily			Not found					
HSP110 family	HSP110/SSE subfamily			Ci-HSPA4/4L/HSPH1	ci0100135787	270	CLSTR03082r1	NP_055093_HS_HSPA4L	<–>
	HSPA14 subfamily			Not found					
	GRP170 subfamily			Ci-HYOU1	c0100147676	24	CLSTR31270r1	NP_006380_HS_HYOU1	<–>
HSPA12 family				Ci-HSPA12	ci0100130734	281	CLSTR05039r1	XP_048898_HS_HSPA12A	<–>

[a] The best-hit model in the version 4 assembly.
[b] The number of scaffold that contains the given gene in the version 4 assembly.
[c] The ID of cDNA cluster in the cDNA/EST database.
[d] The best-hit protein in the human proteome.
[e] "<–>" indicates a bidirectional best-hit relationship between a *Ciona* and a human protein. "–>" indicates a unidirectional best-hit relationship of a *Ciona* protein against a human protein.

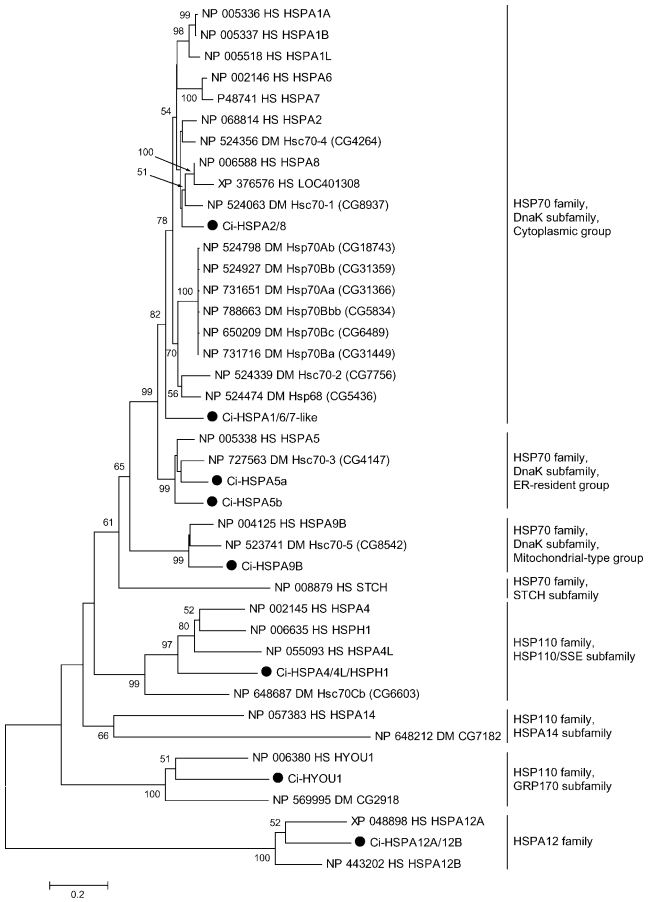

Fig 1. Phylogenetic tree of HSP70 superfamily proteins constructed based on the full-length sequences of HSP70 superfamily proteins of humans, *C intestinalis*, and *D melanogaster*. *Ciona* proteins are indicated by large black dots. The number at each branch indicates the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. Percentages <49% are omitted for simplicity. Proteins are named as explained in Materials and Methods. The unrooted tree is shown as a rooted tree for simplicity. The scale bar indicates an evolutionary distance of 0.2 amino acid substitutions per position. Bars on the right indicate gene groups. As for members of cytoplasmic group of DnaK subfamily, another tree was constructed to know the relationship among them more precisely (supplementary Fig S1).

suggests that 6 clades (the cytoplasmic group of the DnaK subfamily of the HSP70 family, ER-resident group of the DnaK subfamily of the HSP70 family, mitochondrial-type group of the DnaK subfamily of the HSP70 family, HSP110/SSE subfamily of the HSP110 family, GRP170 subfamily of the HSP110 family, and the HSPA12 family) contain both human and *Ciona* members (Fig 1). Every clade except for the HSPA12 family also contains *Drosophila* members. Notably, the cytoplasmic group of the DnaK subfamily contains only 2 *Ciona* proteins, whereas 10 *Drosophila* and 8 human proteins belong to this group (Fig 1). The cytoplasmic group of the DnaK subfamily consists of HSPA2/8 subgroup, HSPA6/7 subgroup, HSPA1 subgroup, and *Drosophila*-specific cytoplasmic HSP70 subgroup (supplementary Fig S1). One of the 2 *Ciona* proteins was assigned into HSPA2/8 subgroup and the other was located outside of the 4 clades and thus the gene for this protein was named Ci-HSPA1/6/7–like (Table 1; supplementary Fig S1). As for the STCH subfamily of the HSP70 family and HSPA14 subfamily of the HSP110 family, no candidate gene was found by the present survey, although each subfamily contains both vertebrate and protostome members.

J-proteins

Members of the J-protein family are characterized by the presence of a conserved domain called the J-domain (Walsh et al 2004). The J-domain consists of approximately 70–75 amino acid residues that form 4 helices with the invariant tripeptide HPD between the second and third helices, and is essential for J-proteins to stimulate ATP hydrolysis by HSP70s (Wall et al 1994; Tsai and Douglas 1996).

A system for classification of the J-proteins according to their structure was proposed (Cheetham and Caplan 1998) and then partly modified (Ohtsuka and Hata 2000). In the proposed system, the J-proteins are categorized into 3 groups: type A (or type I) proteins have all of the J-domain, the glycine- and phenylalanine-rich region, and the DnaJ central domain; type B (or type II) proteins have the J-domain and the glycine- and phenylalanine-rich region but not the DnaJ central domain; and type C (or type III) proteins have the J-domain but lack the other 2 domains found in type A. Members of each type are numbered according to the chronological order of registration of the sequence data (Cheetham and Caplan 1998; Ohtsuka and Hata 2000).

Fifty human J-proteins are registered in the public databases. Among them, 5 (DNAJA1–DNAJA5), 11 (DNAJB1–DNAJB9, DNAJB11, and DNAJB12), and 16 (DNAJC1–DNAJC5, DNAJC5beta, DNAJC5gamma, and DNAJC6–DNAJC14) proteins have been categorized as type A, type B, and type C, respectively, and have been designated systematically. The other 17 proteins have not been classified into any of these 3 types and have been designated in a nonsystematic manner. Our present analysis suggested that the J-domain sequences of 4 of the J-proteins (LOC387820, FLJ14281, MGC29463, and TSARG6) show similarity to those of type B proteins; hence they were classified into type B. Another 14 proteins (GAK, DNAJD1, TIM14, SEC63, RBJ, HSC20, WBSCR18, sacsin, GNG10, ZCSL3, C21orf55, KIAA0962, FLJ13236, and FLJ10634) were regarded as type C proteins.

Very recently, identification of 15 J-protein–encoding genes in the *C intestinalis* genome was reported (Satouh et al 2005). In the present survey, we identified 36 *Ciona*

genes for J-proteins, including all of the 15 genes reported in that paper. The phylogenetic analyses suggest that 36 *Ciona* and 50 human genes for J-proteins are classified into 33 groups along with 3 orphan genes specific to *Ciona* (see Table 2; supplementary Table S3; Fig 2; supplementary Figs S2–S4). Among the 33 groups, 28 groups contain both human and *Ciona* proteins, whereas the other 5 groups (DNAJC14, ZCSL3, C21orf55, FLJ13236, and sacsin groups) contain only human proteins. All of the 33 groups except for RBJ and sacsin groups contain protostome members (data not shown). Thus, the sacsin group seems to be vertebrate specific. In addition to 36 genes for true J-proteins, we identified a gene for a J-like protein that lacks the HPD tripeptide motif but showed similarity to type A J-proteins (Ci-DnaJ–like) (Table 2; Table S3; supplementary Fig S5).

BAG family proteins

The BAG domain is a conserved domain that comprises about 50 amino acid residues and is shared by 6 human BAG family proteins: BAG1, BAG2, BAG3, BAG4, BAG5, and BAT3 (Takayama and Reed 2001; Doong et al 2002; Alberti et al 2003). The BAG domain of BAG1, BAG2, BAG3, and BAG4 has been shown to bind to HSP70s, and the BAG family proteins are thought to be involved in the regulation of HSP70s. In protostomes, BAG1, BAG2, BAG3, and BAT3 group proteins have been identified. The present survey identified 3 genes for BAG family proteins (Ci-BAG1, Ci-BAG3, and Ci-BAT3) in the genome of *C intestinalis* (Table 3; supplementary Table S4). The results of best-hit analyses and comparisons of protein sequences suggest that Ci-BAG1, Ci-BAG3, and Ci-BAT3 belong to BAG1, BAG3, and BAT3 groups, respectively (Table 3; supplementary Figs S6–S8). As for BAG2, BAG4, and BAG5 groups, no candidate gene was found by the present survey. Genes of BAG4 and BAG5 groups seem to be vertebrate specific.

Other types of cochaperones

In addition to J-proteins and BAG family proteins, several proteins with different structural properties were shown to act as cochaperones of HSP70s. A significant fraction of such cochaperones is characterized by the presence of the TPR domain (Blatch and Lassle 1999). However, TPR domain–containing cochaperones for HSP70s in *Ciona* will be described in our upcoming paper that reports HSP90 family proteins and their cochaperones (Hamada et al, in preparation), because some of them also interact with HSP90s. Therefore, cochaperones for HSP70s without the TPR domain were subjected to the present analysis and are described in this section. We searched for genes of following groups in the *Ciona* genome: SIL1 (BAP) (Tyson and Stirling 2000; Chung et al 2002), HSPBP1 (Hsp70 binding protein 1) (Raynes and Guerriero 1998; Kabani et al 2002), UBQLN (Ubiquilin) (Kaye et al 2000), UBADC1 (Ubiquilin associated domain containing 1) (Li et al 2000), TIMM44, GRPEL, and Magmas (Koehler 2004; Wiedemann et al 2004). For all of these groups, there are both human and protostome members. The present survey identified single members in *Ciona* for each group except for HSPBP1 (Ci-SIL1, Ci-UBQLN, Ci-UBADC1, Ci-TIMM44, Ci-GRPEL, and Ci-Magmas) (see Table 4; supplementary Table S5; supplementary Figs S9 and S10). Ci-Magmas is identical to the recently reported magmas-like protein of *C intestinalis* (Peng et al 2005).

DISCUSSION

Components of the HSP70 chaperone system are highly conserved but different among vertebrates, *Ciona*, and protostomes

In the present study, we searched for members of the HSP70 superfamily, J-proteins, BAG family, and other types of cochaperone (SIL1, HSPBP1, UBQLN, UBADC1, TIMM44, GRPEL, and Magmas) in *C intestinalis*. The results of the present study show that genes of the HSP70 chaperone system can be distinguished into groups that are shared by vertebrates, *Ciona*, and protostomes, ones shared by vertebrates and protostomes, ones shared by vertebrates and *Ciona*, and ones specific to vertebrates, *Ciona*, or protostomes.

Many of gene groups examined in the present study (42 groups of 54 groups) are shared by vertebrates, *Ciona*, and protostomes. This confirms that the components of the HSP70 chaperone system are highly conserved among metazoans and suggests that these gene groups are essential for function of the HSP70 chaperone system in metazoans.

Genes of 8 groups are found in both vertebrates and protostomes but not identified in *Ciona* (STCH, HSPA14, DNAJC14, ZCSL3, C21orf55, FLJ13236, BAG2, and HSPBP1 groups). Those genes may have been lost in the lineage leading to ascidians after the separation from that leading to vertebrates. Alternatively, because the published sequence of the *Ciona* genome was estimated to contain about 95% of genes that are present in the genome (Dehal et al 2002), those genes may be located in regions of the genome that have not been sequenced. However, if that is the case, those genes should be rarely transcribed or be unstable in cDNA libraries, because we searched for the cDNA database as well. Another possibility is that sequences of those genes have changed extensively during evolution of *Ciona* so that we are not able to recognize them.

Among gene groups examined in the present study, the

Table 2 Genes for J-proteins in the *C intestinalis* genome

Type	Group	Gene name	The best gene model[a]	Scaffold[b]	cDNA cluster[c]	The best-hit protein[d]	Best-hit analysis[e]
Type A	DNAJA1/2/4 group	Ci-DNAJA1/2/4	ci0100134317	2	CLSTR01069r1	NP_001530_HS_DNAJA1	<–>
	DNAJA3 group	Ci-DNAJA3	ci0100148427	132	CLSTR05897r1	NP_005138_HS_DNAJA3	<–>
	DNAJA5 group	Ci-DNAJA5	ci0100149231	826	CLSTR08795r1	NP_001012339_HS_DNAJA5 isoform 2	<–>
Type B	DNAJB1/4/5 group	Ci-DNAJB1/4/5	ci0100130510	141	CLSTR07390r1	NP_03698_HS_DNAJB5	–>
	DNAJB2/3/6/7/8 group	Ci-DNAJB2/3/6/7/8	ci0100138562	491	CLSTR08173r1	NP_490647_HS_DNAJB6	<–>
	DNAJB9 group	Ci-DNAJB9	ci0100139615	454	CLSTR12988r1	NP_036460_HS_DNAJB9	–>
	DNAJB11 group	Ci-DNAJB11	ci0100137275	448	CLSTR01142r1	NP_057390_HS_DNAJB11	<–>
	DNAJB12 group	Ci-DNAJB12/FIJ14281/ MGC29463	ci0100147080	113	CLSTR01694r1	NP_001002762_HS_DNAJB12	<–>
	TSARG6 group	Ci-TSARG6 (CiAxHsp40)	ci0100135431	649	CLSTR10996r1	NP_705842_HS_TSARG6	<–>
Type C	DNAJC1/2 group	Ci-DNAJC1	ci0100150239	401	CLSTR04059r1	NP_071760_HS_DNAJC1	<–>
		Ci-DNAJC2	ci0100138352	126	CLSTR03143r1	XPII379909_HS_DNAJC2	<–>
	DNAJC3/7 group	Ci-DNAJC3	ci0100135758	339	CLSTR05227r1	NP_006251_HS_DNAJC3	<–>
		Ci-DNAJC7	ci0100130790*	367	CLSTR12290r1	NP_003306_HS_DNAJC7	<–>
	DNAJC4 group	Ci-DNAJC4a	ci0100140733	375	CLSTR05848r1	NP_005138_HS_DNAJA3	–>
		Ci-DNAJC4b	ci0100131740*	375	CLSTR07706r1	NP_149096_HS_DNAJC5B	–>
		Ci-DNAJC4c	ci0100131740*	375	CLSTR03807r1*	NP_001530_HS_DNAJA1	–>
		Ci-DNAJC4d	None	375	CLSTR03807r1*	NP_660157_HS_DNAJB7	–>
	DNAJC5 group	Ci-DNAJC5/5G/5B	ci0100143085	46	CLSTR01145r1	NP_079495_HS_DNAJC5	<–>
	DNAJC6/GAK group	Ci-DNAJC6/GAK	ci0100139892	11	CLSTR02042r1	NP_005246_HS_GAK	<–>
	DNAJC8 group	Ci-DNAJC8	ci0100134649	73	CLSTR08118r1	NP_055095_HS_DNAJC8	<–>
	DNAJC9 group	Ci-DNAJC9	ci0100137534	85	CLSTR12608r1	NP_056005_HS_DNAJC9	<–>
	DNAJC10 group	Ci-DNAJC10	ci0100137316	319	CLSTR01755r1	NP_061854_HS_DNAJC10	<–>
	DNAJC11 group	Ci-DNAJC11	ci0100145140	26	CLSTR7022r1	NP_060668_HS_DNAJC11	<–>
	DNAJC12 group	Ci-DNAJC12	ci0100145102	240	CLSTR12602r1	NP_068572_HS_DNAJC12	<–>
	DNAJC13 group	Ci-DNAJC13	ci0100135615	337	CLSTR01643r1	NP_776184_HS_DNAJC13	<–>
	DNAJD1/TIM14 group	Ci-DNAJD1/TIM14	ci0100131878*	721	CLSTR12593r1	NP_660304_HS_TIM14 isoform a	<–>
	SEC63 group	Ci-SEC63	ci0100154010	437	CLSTR00426r1	NP_009145_HS_SEC63	<–>
	RJL group	Ci-RBJ	ci0100131065	5	CLSTR10536r1	NP_057628_HS_RBJ	<–>
	HSC20 group	Ci-HSC20	ci0100130254*	38	CLSTR33919r1	NP_741999_HS_HSC20	<–>
	KIAA0962 group	Ci-KIAA0962	ci0100131698	313	CLSTR16673r1	NP_056106_HS_KIAA0962	<–>
	GNG10 group	Ci-GNG10	ci0100149135	1	CLSTR04741r1	NP_001015882_HS_GNG10	<–>
	WBSCR18 group	Ci-WBSCR18	ci0100154690	310	CLSTR00516r1	NP_115693_HS_WBSCR18	–>
	FIJ10634 group	Ci-FLJ10634	ci0100139365	254	CLSTR03751r1	NP_060633_HS_FIJ10634	<–>
	DNAJC14 group	Not found					
	ZCSL3 group	Not found					
	C21orf55 group	Not found					
	FIJ13236 group	Not found					
	Sacsin group	Not found					
Orphan genes		Ci-orphan-DnaJ1	ci0100144347	48	CLSTR05620r1	NP_001530_HS_DNAJA1	–>
		Ci-orphan-DnaJ2	ci0100153801	214 (3951)	CLSTR12278r1	NP_079495_HS_DNAJC5	–>
		Ci-orphan-DnaJ3	ci0100143483	195 (3768)	CLSTR37398r1	NP_061854_HS_DNAJC10	–>
DnaJ-like gene		Ci-DnaJ-like	ci0100142591	35	CLSTR12882r1	NP_001530_HS_DNAJA1	–>

[a] The best-hit model in the version 4 assembly. The models marked by asterisk contain two or more actually distinct genes improperly.
[b] The number of scaffold that contains the given gene in the version 4 assembly.
[c] The ID of cDNA cluster in the cDNA/EST database. CLSTR03807r1 (marked by asterisk) contains ESTs of 2 genes (Ci-DNAJC4b and Ci-DNAJC4c).
[d] The best-hit protein in the human proteome.
[e] "<–>" indicates a bidirectional best-hit relationship between a *Ciona* and a human protein. "–>" indicates a unidirectional best-hit relationship of a *Ciona* protein against a human protein.

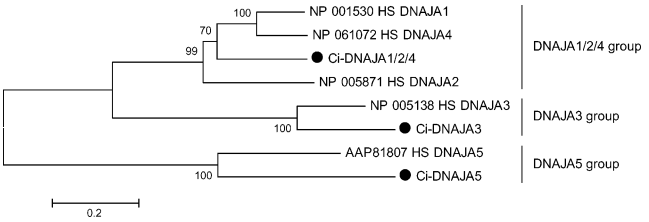

Fig 2. Phylogenetic tree of type A J-proteins constructed based on the full-length sequences of type A J-proteins of humans and *C intestinalis*. *Ciona* proteins are indicated by large black dots. The number at each branch indicates the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. Proteins are named as explained in Materials and Methods. Bars on the right indicate gene groups. The unrooted tree is shown as a rooted tree for simplicity. The scale bar indicates an evolutionary distance of 0.2 amino acid substitutions per position.

RBJ group of J-protein family is the only group that is found in vertebrates and *Ciona* but not in protostomes. Thus, the RBJ group may be related to a deuterostome-specific or chordate-specific innovation. Proteins of the RBJ group belong to the Ras superfamily of GTP binding proteins and have a Ras-related GTPase domain in addition to the J-domain (Nepomuceno-Silva et al 2004). In vertebrates, RBJ group genes are predominantly expressed in the nervous system, and it has been suggested that they are involved in the development or maintenance of the sophisticated chordate nervous system (Nepomuceno-Silva et al 2004).

Three gene groups (sacsin group of the J-protein family and BAG4 and BAG5 groups of BAG family) were found in neither *Ciona* nor protostomes and seem to be specific to vertebrates. Although the function of BAG5 is undetermined, known functions of sacsin and BAG4 suggest that they may be related to vertebrate-specific innovations. Human sacsin has been identified as a gene responsible for a human genetic neurodegenerative disease called ARSACS (autosomal recessive spastic ataxial of Charlevoix-Saguenary), which is involved in a developmental defect in myelination of both retinal and peripheral nerve fibers (Engert et al 2000). Expression of sacsin mRNA is detected in the entire central nervous system of the developing and adult rat (Engert et al 2000). The fact that ARSACS is involved in abnormal myelination process is interesting, because myelin sheaths occur in gnathostomes but not in agnathans and invertebrates. It is possible that sacsin has emerged in gnathostome lineage during evolution in accord with the emergence of myelin sheaths.

BAG4 was identified as a protein that binds to the tumor necrosis factor receptor 1 (TNFR1) and regulates the activity of TNFR1 (Jiang et al 1999). BAG4 recognizes the death domain of TNFR1 (Jiang et al 1999). It has been suggested that vertebrate TNFR superfamily proteins have acquired the death domain during evolution (Bridgham et al 2003). In *Drosophila*, a single gene encoding a protein related to vertebrate TNFR superfamily proteins was identified, and this protein lacks the death domain (Kanda et al 2002; Kauppila et al 2003). In *Ciona*, 3 genes each encoding a protein related to vertebrate TNFR superfamily proteins were identified (Terajima et al 2003). Similar to the TNFR-related protein in *Drosophila*, all *Ciona* TNFR-related proteins lack the death domain. Therefore, it is likely that the emergence of both BAG4 and TNFR superfamily proteins with the death domain has occurred in the lineage leading to vertebrates after the separation from that leading to ascidians.

Interestingly, the contribution of the TNF signaling in apoptosis differs between mammals and *Drosophila*. In mammals, the TNF signaling activates 3 distinct pathways (FADD/caspase-8–mediated pathway, NF-κB–mediated pathway, and JNK-mediated pathway), whereas only the JNK-mediated pathway is downstream to the TNF signaling in *Drosophila* (Moreno et al 2002). It has been suggested that the presence of the death domain in mammalian TNFR superfamily proteins accounts for such difference (Bridgham et al 2003). Thus, the emergence of BAG4 may be related to the death domain–mediated functional diversification of the TNF signaling.

Table 3 Genes for BAG-family proteins in the *C intestinalis* genome

Group	Gene name	The best gene model[a]	Scaffold[b]	cDNA cluster[c]	The best-hit protein[d]	Best-hit analysis[e]
BAG1 group	Ci-BAG1	ci0100140120	22	CLSTR03639r1	NP_004314_HS_BAG1L	<–>
BAG2 group	Not found					
BAG3 group	Ci-BAG3	ci0100139061	11	CLSTR01615r1	NP_004272_HS_BAG3	<–>
BAG4 group	Not found					
BAG5 group	Not found					
BAT3 group	Ci-BAT3	ci0100130473	134	CLSTR03629r1	NP_004630_HS_BAT3	<–>

[a] The best-hit model in the version 4 assembly.
[b] The number of scaffold that contains the given gene in the version 4 assembly.
[c] The ID of cDNA cluster in the cDNA/EST database.
[d] The best-hit protein in the human proteome.
[e] "<–>" indicates a bidirectional best-hit relationship between a *Ciona* and a human protein.

Table 4 Genes for other types of cochaperones for HSP70s in the *C intestinalis* genome

Group	Gene name	The best gene model[a]	Scaffold[b]	cDNA cluster[c]	The best-hit protein[d]	Best-hit analysis[e]
SIL1 (BAP) group	Ci-SIL1	ci0100152004	1049	CLSTR15579r1	NP_071909_HS_SIL1	<–>
HSPBP1 group	not found					<
UBQLN group	Ci-UBQLN	ci0100143699	26	CLSTR09463r1	NP_048466_HS_UBQLN1 isoform 1	>
UBADC1 group	Ci-UBADC1	ci0100140731	752	CLSTR06774r1	NP_057256_HS_UBACD1	<–>
TIMM44 group	Ci-TIMM44	ci0100143454	16	CLSTR05859r1	NP_006342_HS_TIMM44	<–>
GRPEL group	Ci-GRPEL	ci0100136893	845	CLSTR03165r1	NP_079472_HS_GRPEL1	<–>
Magmas group	Ci-Magmas	ci0100146480	30	CLSTR05000r1	NP_057153_HS_Magmas	<–>

[a] The best-hit model in the version 4 assembly.
[b] The number of scaffold that contains the given gene in the version 4 assembly.
[c] The ID of cDNA cluster in the cDNA/EST database.
[d] The best-hit protein in the human proteome.
[e] "<–>" indicates a bidirectional best-hit relationship between a *Ciona* and a human protein.

Putative evolutionary events that led to differences in the components of the HSP70 chaperone system between *Ciona* and humans

The results of the present study provide a light on the evolutionary process that resulted in similar but different HSP70 chaperone systems in *Ciona* and humans, as discussed below.

The number of HSP70 superfamily proteins in *Ciona* is about one-third of that in humans. This is due in part to the fact that multiple members for 3 of the HSP70 superfamily in humans are represented by a single protein in *Ciona* (ER-resident group of the DnaK subfamily of the HSP70 family, cytoplasmic HSP110 subfamily of the HSP110 family and HSPA12 family). Similarly, the number of proteins in the cytoplasmic group of the DnaK subfamily of the HSP70 family in *Ciona* is much smaller than that in humans (2 in *Ciona* vs 8 in humans). It seems unlikely that there is another member of this group in regions of the *Ciona* genome that have not been sequenced, because, as mentioned above, the published sequence was estimated to contain about 95% of genes that are present in the *Ciona* genome. Therefore, it is likely that the amplification of members of this group, which appears to have occurred in many lineages, including those leading to lineages of yeasts, *Drosophila*, *C elegans*, fish, and mammals, did not occur or was not retained in the *Ciona* lineage. It has been proposed that HSP70 genes are targets of natural selection and that the amplification of HSP70 genes in *Drosophila* has facilitated the evolution of thermotolerance and niche expansion of this species (Feder and Krebs 1998). In this respect, whether and how environmental factors have influenced the number of HSP70 genes in *Ciona* is an interesting issue for future studies.

There are 36 J-proteins in *Ciona*, whereas there are 50 in humans. Similar to the case of HSP70s, this difference is due in part to the fact that multiple members for 7 groups of J-proteins in humans are represented by a single protein in *Ciona* (DNAJA1/2/4, DNAJB1/4/5, DNAJB2/3/6/7/8, DNAJB12, DNAJC5, DNAJC6/GAK, and DNAJD1/TIM15 groups). As for the UBQLN and GRPEL groups, humans have multiple members but *Ciona* has a single one. These results also suggest that the amplification of genes and/or genomic regions in the lineage leading to humans after the separation from that leading to *Ciona* increased the number and diversity of members of the HSP70 chaperone system.

Gene amplification events seem to have occurred in 2 gene groups in *Ciona* as well (ER-resident group of DnaK subfamily of HSP70 family and DNAJC4 group of J-protein family). As for the ER-resident group of DnaK subfamily, the *Ciona* genome contains 2 genes (Ci-HSPA5a and Ci-HSPA5b), whereas the human genome contains 1 gene. Ci-HSPA5a and Ci-HSPA5b are located on the same

scaffold, although the distance of the 2 genes is over 250 kb. Analysis of ESTs showed that Ci-HSPA5a is actively transcribed, whereas Ci-HSPA5b is not transcribed as far as it was examined by the cDNA project (data not shown). However, the predicted protein sequence of Ci-HSPA5b shows overall similarity to the typical members of the ER-resident group of DnaK subfamily, although it lacks the ER retention signal at the C terminus. Further analysis is required to distinguish whether Ci-HSPA5b is a pseudogene or not. Although there are number of HSP70 pseudogenes (ie, sequences that encode fragmentary HSP70 superfamily protein) in the human genome, no such sequence was found in the *Ciona* genome.

The *Ciona* genome contains 4 genes of the DNAJC4 group of J-protein family, whereas the human genome contains 1 gene of this group. Four DNAJC4 group genes in *Ciona* are aligned in tandem in the same scaffold, suggesting that they are products of local gene duplication events. Analysis of ESTs suggests that every gene is transcribed (data not shown). The significance of the amplification of DNAJC4 group genes in *Ciona* remains to be investigated.

There are 3 orphan genes encoding J-proteins and a gene encoding J-like protein in the *Ciona* genome. It is likely that these genes have emerged in the lineage leading to *Ciona* after the separation from that leading to humans, because there are no counterparts for these genes in protostomes as well. Similar to the case of gene groups that are conserved in vertebrates and protostomes but not in *Ciona*, there is alternative possibility that sequences of those genes have changed extensively in during evolution of both vertebrates and protostomes so that we are not able to recognize them.

In summary, the results of the present study suggest that the difference in the components of the HSP70 chaperone system between humans and *Ciona* has originated form the following events: the vertebrate-specific innovation of 1 group (sacsin group), the gene amplification in humans in 13 groups (cytoplasmic group of the DnaK subfamily, ER-resident group of the DnaK subfamily, cytoplasmic HSP110 subfamily of the HSP110 family, HSPA12 family, DNAJA1/2/4, DNAJB1/4/5, DNAJB2/3/6/7/8, DNAJB12, DNAJC5, DNAJC6/GAK, DNAJD1/TIM15, UBQLN, and GRPEL groups), the gene loss in *Ciona* in 8 groups (STCH, HSPA14, DNAJC14, ZCSL3, C21orf55, FLJ13236, BAG2, and HSPBP1 groups), the gene amplification in *Ciona* in 2 groups (ER-resident group of DnaK subfamily of HSP70 family and DNAJC4 group of J-protein family), and the occurrence of 4 genes unique to *Ciona* (3 orphan genes encoding J-proteins and a gene encoding J-like protein). The difference in the variety of the components of the HSP70 chaperone system may be related to the difference in the ability of stress response between humans and *Ciona*. Studies on stress response in *Ciona* and function of *Ciona* genes identified in this study will be helpful to test this idea.

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