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MINIREVIEW

The Clp Proteins: Proteolysis Regulators or Molecular Chaperones?

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INTRODUCTION

The Clp proteins constitute a recently discovered family that is represented in every organism examined thus far. (In this review we use the name Clp to mean the family of large homologous proteins, ClpA to -C [756 to 926 amino acids]. This family is distinct from the nonhomologous protease ClpP [207 amino acids].) The family comprises at least three subfamilies, and although they differ somewhat in size and sequence organization, about 420 amino acids in two extensive sequence blocks have been very highly conserved (14). It is probable not only that the family is universal but that several different kinds of Clp proteins (Clp subfamilies) can be made by all cells. For example, *Escherichia coli* possesses two large Clp proteins, ClpA and ClpB (13, 41), and both human cells and Chinese hamster cells possess multiple proteins that react with antibodies to hsp104, a Clp present in *Saccharomyces cerevisiae* (37). Investigations with *E. coli* ClpA suggest that it is involved in the regulation of proteolysis because ClpA strongly activates the nonhomologous ClpP peptidase in the ATP-dependent cleavage of denatured proteins (18, 21). The yeast Clp is the heat shock protein hsp104 (37), and the *E. coli* ClpB (23, 37, 41) is the previously identified heat shock protein F84.1 (41). Many heat shock proteins are molecular chaperones (7), and it has been suggested that rather than operating strictly as proteolytic regulators, the heat shock Clp proteins function as chaperones that protect vital cellular components from high-temperature stress (37). This view of Clp protein function is, in fact, not inconsistent with a proteolytic role, since one role of molecular chaperones is to remove denatured proteins which are toxic to the cell. This interpretation does, however, place a different emphasis on the quest for the substrate(s) of the Clp proteins, and it calls for different experimental strategies. The purpose of this review is to summarize what is known about the conservation and structure of Clp proteins and to evaluate the evidence that pertains to their function.

ORGANIZATION AND CONSERVATION

Comparison of Clp sequences from different organisms has established the relatedness of family members and has revealed at least three subfamilies, ClpA, ClpB, and ClpC. Comparisons also provide clues about the function of the family. Altogether, nine complete and three fragmentary Clp sequences have been obtained from 10 different organisms (Table 1). Additional unpublished sequences and experiments with Clp probes increase the number of observations and suggest that the family is represented in all three kingdoms. Alignment of the 10 sequences reveals that all contain two nucleotide-binding regions (N1 and N2) separated by a variable spacer sequence (S) and enclosed between somewhat more variable leader (L) and trailer (T) sequences (Fig. 1 and Table 1). In the following paragraphs, we will discuss new observations concerning the nucleotide-binding regions and the leader region and will summarize what is known about conservation and the subfamilies of Clp proteins.

The two nucleotide-binding sites are necessary for function. The nucleotide-binding sites are a prominent feature of the dissimilar N1 and N2 sequences, and both are necessary for function of the yeast ClpB gene, *HSP104*. These sites contain classical two-part nucleoside triphosphate-binding consensus sequences consisting of a glycine-rich segment A followed after approximately 60 amino acids by a hydrophobic segment B (5, 44) (Fig. 1). Changes that destroy the segment A consensus sequence of either N1 or N2 abolish the yeast thermotolerance response (37). ATPase activities have been demonstrated in vitro for *E. coli* ClpA (18–20) and ClpB (11, 29) and for yeast hsp104 (27).

Proteins that contain two nucleotide-binding sites are rare. The only other examples are some of the traffic ATPases, proteins that provide energy for permeation (1, 2). However, the sequences surrounding the two nucleotide-binding sites in traffic ATPases are clearly related to each other, while N1 and N2 in Clp proteins are nonhomologous. This analogy with traffic ATPases does, however, focus attention on the idea that Clp proteins might be a fusion of two dissimilar nucleotide-binding proteins. An exhaustive search for other proteins that are similar to either N1 or N2 found no homologs. The closest similarity to other proteins is found spanning the segment A consensus sequence. Parsell et al. have noted that the amino acids that include this region from N1 are like the β-subunit F_1 ATPase, while the same region from N2 more closely resembles the myosin nucleotide-binding site (37). These similarities, however, do not extend to the segment B consensus sequences, nor do any other regions of N1 or N2 show similarities to the ATPase or myosin sequences.

The leader region: transit sequences and a conserved tandem duplication. Clp leader sequences are relatively uniform in length, with the exceptions of the tomato (14) and pea (33) ClpC sequences, which are about 100 amino acids longer than the rest. Recent experiments showed that the pea ClpC is transported into chloroplasts with concomitant loss of a 90- to 100-amino-acid N-terminal transit sequence (33). The removal of 91 or 92 residues from the tomato and pea ClpC sequences would make their sizes similar to those of other ClpC leader sequences and identical to the size of the *Mycobacterium leprae* ClpC leader. The similarity of the proposed mature plant and *M. leprae* leader sequences is, indeed, remarkable, with 86% similar and 59% identical amino acids, including six identical N-terminal amino acids.

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TABLE 1. Sizes of the five Clp protein regions[a]

Organism (reference)	Size (amino acids)						Subfamily
	Total	L	N1	S	N2	T	
True bacteria							
Bacteroides nodosus (28)	860	178	233	124	192	133	ClpB
Escherichia coli ClpA (13)	756	180	233	5	189	149	ClpA
Escherichia coli ClpB (41)	857	174	233	124	192	134	ClpB
Escherichia coli ClpB′ (41)	709	26	233	124	192	134	ClpB
Mycobacterium leprae (35)	649[b]	184	232	62	171[b]	—[c]	ClpC
Rhodopseudomonas blastica (43)	793	212	233	5	189	154	ClpA
Streptococcus mutans (14, 39)	289[b]	—	—	—	132[b]	130[b]	ClpB
Eukaryotes							
Arabidopsis thaliana (17)	124[b]	124[b]	—	—	—	—	ClpC
Lycopersicon esculentum CD4A (14)	926	270	232	69	192	163	ClpC
Lycopersicon esculentum CD4B (14)	923	268	232	68	192	163	ClpC
Pisum sativum (33)	922	268	232	68	192	162	ClpC
Saccharomyces cerevisiae (37)	908	180	229	131	192	177	ClpB
Trypanosoma brucei (14)	862	166	233	123	192	148	ClpB

[a] In all cases, amino acid sequences have been predicted from DNA sequence data.
[b] Incomplete sequence.
[c] —, sequence not determined.

Another similarity is shared by all Clp leader sequences. Alignment of the truncated plant ClpC proteins (the sequences without the transit peptide) with the other Clp leader sequences shows a conserved tandem duplication of 32 amino acids, located 18 and 93 amino acids from the N terminus of the *M. leprae*, tomato, and pea sequences (Fig. 1). These duplicated regions are very similar within the ClpC subfamily, and although the sequences have drifted in the ClpA and ClpB examples, homologies at the same two locations can be identified by computer alignment (42). The function of the duplicated sequences is not known, but they might also be involved in mobilizing Clp proteins for transport. Potential signal sequences are associated with both duplications in every example. In other proteins, multiple signal sequences are involved in sequential transport through several membrane barriers (22). Alternatively, insertion of two hydrophobic helices into the membrane, without subsequent transport and cleavage, might anchor Clp to the membrane. With the exception of plant ClpC, possible transport and membrane affinities of Clp proteins have not yet been investigated.

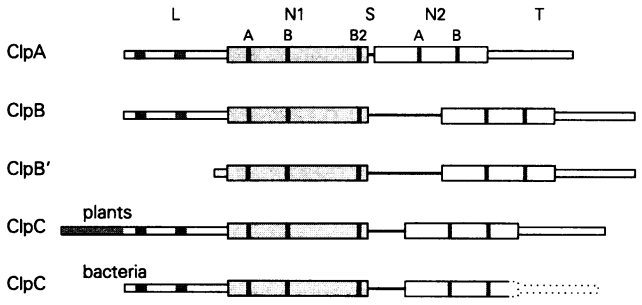

FIG. 1. Schematic of large Clp proteins, showing regions of varying conservation. Thicker boxes represent more conserved parts of the proteins. Regions of the proteins are identified as leader (L), spacer (S), and trailer (T), delimiting the two highly conserved nucleotide-binding sites (N1 and N2), which contain segment A and B consensus sequences (5, 44). The N1 region also contains a second segment B (B2). Short duplicate sequences in the leader regions are shaded. The longer shaded region in the ClpC leader from plants is the chloroplast transit sequence. ClpB′ is a truncated molecule that is made together with the full-length ClpB in *E. coli* by using an alternate translational initiation site at codon 149. The sequences are only approximately to scale; exact sizes are given in Table 1. Dotted lines indicate that the sequence is incomplete.

Interest in the tandem duplication has increased with the discovery that two proteins, ClpB and ClpB′, are made from the *clpB* gene in *E. coli* (41) (Fig. 1). ClpB contains the duplicated region while ClpB′ does not, because ClpB′ translation commences at a second start codon 149 codons beyond the ClpB start. The presence of two forms of ClpB in *E. coli* cells might provide a means of targeting the protein to separate tasks, possibly by a mechanism similar to that used by *S. cerevisiae* to direct its *MOD5* gene product (a tRNA-modifying enzyme) to both mitochondria and the cytosol. This gene also makes two isoenzymes from one gene by alternate translational initiation (9). The longer product has a transit sequence and is targeted to the mitochondrion, while the shorter product remains in the cytosol.

Conservation and subfamily relatedness. Both N1 (229 to 233 amino acids) and N2 (189 to 192 amino acids) sequences are very highly conserved. Every N1 or N2 sequence shares at least 50% identical and 85% similar amino acids with its most distant relative (14). This level of identity is remarkable, and it is therefore noteworthy that the ClpA and ClpB homologs from *E. coli* are more distantly related than the ClpB sequences of *E. coli* and the trypanosome (14). Initially, subfamilies were assigned arbitrarily according to the size of the spacer region. The ClpA subfamily has a short (5 amino acids) spacer, that of ClpB is the longest (123 to 131 amino acids), and the ClpC spacer is intermediate (62 to 69 amino acids) (Fig. 1). Now it appears that these subfamilies may represent functional groups, since four ClpB examples are heat shock proteins (see heat shock section below). Less information is available concerning possible group functions of ClpC and ClpA. The plant ClpC proteins are transported into chloroplasts (33), but since there is also a prokaryotic ClpC sequence (from *M. leprae*), a strictly chloroplast role is ruled out. The ClpA in *E. coli* is made constitutively at normal temperatures and is involved in regulation of proteolysis, but nothing is known about the other ClpA sequence (from *Rhodopseudomonas blastica*) that can add to this picture.

Several studies suggest that there are multiple *clp* genes and proteins within the same cell, but only for *E. coli* have representatives of two subfamilies been sequenced (13, 41). Experiments with a *clpB* DNA probe showed hybridization with multiple restriction fragments from *S. cerevisiae*, *Drosophila melanogaster*, and the archaebacterium *Methanosarcina acetivorans* DNAs (14), possibly reflecting the presence of multiple Clp subfamilies in other cells. Lindquist and coworkers have shown that yeast hsp104 (ClpB) antibodies react with two human heat shock proteins (100 to 110 kDa) and with four Chinese hamster ovary proteins, two heat inducible (100 to 110 kDa) and two constitutive (70 to 80 kDa) (37).

THE PROTEASE CONNECTION: *E. COLI* ClpA-ClpP

One member of the Clp family, *E. coli* ClpA, has been isolated with a smaller, nonhomologous protein, ClpP; together they carry out ATP-dependent degradation of casein in vitro. Work on the genetics and biochemistry of this protease has been pursued by Gottesman, Maurizi, and coworkers, who have called it the ClpA-ClpP protease, and by Goldberg and coworkers, who refer to the complex as Ti protease. The studies by Goldberg and Gottesman have shown that the actual proteolytic activity of the ClpA-ClpP complex resides in the ClpP subunit (19, 32). ClpA alone has no demonstrable proteolytic activity, but it does have intrinsic ATPase activity (18–20). ClpP can degrade short peptides on its own, but it must be complexed with ClpA to cleave denatured proteins (45). Structural studies of purified ClpA-ClpP suggest that the two subunits form a 750-kDa complex made up of 6 ClpA and 12 ClpP subunits (19, 30). It was suggested that this ClpP structure and its interaction with ClpA provides *E. coli* with a prototype proteasome complex (31). Such proteasomes are thought to be important ATP-dependent proteolytic complexes in eukaryotic cells (3).

Three experimental approaches have been used to examine the in vivo function of ClpA. The first experiment compared the breakdown of abnormal canavanine-containing proteins in protease *lon* and *lon clpA* mutants (20). The *lon* mutation reduced the cell's ability to degrade abnormal proteins; the *lon clpA* mutant had even further reduced proteolytic ability, suggesting that ClpA accounts for part of the abnormal protein degradation. However, the *clpA* mutation alone had little or no effect on degradation of canavanine proteins (20). Second, experiments showed that the half-life of a ClpA–β-galactosidase fusion protein is influenced by mutations in the *clpA* and *clpP* genes. In a $clpA^+$ $clpP^+$ strain the fusion protein was degraded rapidly, while the half-life was longer in *clpA* (13) and *clpP* (31) mutants. It was concluded from these studies that the ClpA protein is, itself, a substrate of the ClpA-ClpP protease (13). Since the ClpA–β-galactosidase fusion protein is degraded considerably faster than the intact ClpA protein, it was suggested that the fusion protein might also be preferentially degraded because it is not folded properly. Third, Varshavsky and coworkers have recently shown that the N-end rule, relating the half-lives of proteins to their N-terminal amino acid, also applies to *E. coli* and that in a *clpA* null mutant the half-life of several test proteins is significantly increased (42a). The authors emphasize that this finding, while placing ClpA in a larger proteolytic system, does not identify the physiologically relevant substrate(s) of ClpA.

Is ClpP associated with ClpB or ClpC? The proteolytic subunit ClpP has been shown to form multimeric complexes only with ClpA. Recent work by Goldberg and Maurizi and coworkers indicates that purified ClpB protein does not substitute for ClpA in supporting proteolytic activity of ClpP (11, 29). On the other hand, there are some intriguing observations that suggest ClpP might be involved with ClpB and ClpC. Members of the ClpB subfamily are heat shock proteins, and in *E. coli*, ClpP has also been identified as the heat shock protein F21.5 (24). Examination of the *E. coli clpP* sequence reveals both σ^{32} and σ^{70} promoters (41), accounting for the ClpP synthesis observed at both normal and high temperatures. In plants, ClpC protein is transported into chloroplasts (33), and it has been noted that a ClpP sequence is encoded on the chloroplast genome (32). ClpP must play an extremely important role in the chloroplast, since it is one of the few genes retained on the abbreviated plastid genome of the nonphotosynthetic plant beechdrops (*Epifagus virginiana*). This parasitic plant possesses a plastid that has lost the photosynthetic and chlororespiratory genes from its genome (6, 34, 36). Most of the remaining genes on this highly deleted genome are involved in translation. These tRNA, rRNA, and ribosomal protein genes have been retained to synthesize at most four proteins that are not part of the gene expression machinery, one of which is ClpP (36). The nonphotosynthetic plastids of *E. virginiana* most probably carry out a number of vital functions for the plant, using ClpP and nucleus-encoded proteins. Presumably ClpC is transported into *Epifagus* plastids just as it is in other plants. These two coincidences underscore the possibility that ClpP subunits associate with ClpB and ClpC under heat shock conditions and in the chloroplasts of plants.

HEAT SHOCK: THE ClpB SUBFAMILY

The heat shock dimension of the Clp story was discovered by Lindquist and coworkers in their studies of yeast hsp104 (38). This work has led to the identification of hsp104 as a ClpB protein (37). *E. coli clpB* is also a heat shock gene (23, 37, 41) that makes the two proteins, F84.1 (ClpB) and F68.5 (ClpB′). The formation of these two proteins is mediated by separate translational initiations and may ensure partitioning of the Clp protein between different locations in the cell or different tasks (41). The yeast work has shown that one hsp104 antibody reacts with mammalian hsp110 (37), perhaps extending the list of Clp heat shock proteins to include this important group.

Negative mutations reveal that the ClpB subfamily is necessary for survival at high temperatures. In *S. cerevisiae*, *HSP104* negative mutations cause a loss of induced thermotolerance and accumulation of protein aggregates (27, 37). In *E. coli*, a *clpB* null mutation results in a slower growth rate at 44°C and an increased death rate at 50°C (41), but in neither case is the precise function of the ClpB known. A careful search for possible defects in proteolysis caused by yeast *HSP104* mutations found no effect (27, 37). Attempts to demonstrate a ClpB influence on proteolysis in *E. coli* either in vivo or in vitro have likewise been negative (11, 29). Because the phenotype caused by *HSP104* mutations is partially suppressed by overproduction of hsp70, Lindquist and coworkers have suggested that Clp proteins protect the cell from heat stress by preventing denaturation of essential cellular components or by rescuing components that have been denatured. If *HSP104* mutations do influence proteolysis, they may do so through more general influences on "disaggregation or denaturation activity" (37).

As mentioned above, there is evidence linking all of the complete ClpB sequence examples to heat shock. Two pieces of evidence suggest that *Trypanosoma brucei* ClpB is heat inducible. *T. brucei* grows in two hosts, the tsetse fly and mammals; these distinct life-cycle stages have temperature

optima at 27 and 37°C, respectively. Heat shock is induced in organisms isolated from the insect vector following a shift to 37°C (10), and Carrington has found that the amount of ClpB mRNA is at least 10-fold greater in the mammalian stage (37°C) than it is in the insect stage (27°C) (4). This conclusion is further supported by the observation (37) that the *T. brucei* ClpB sequence ends with a C-terminal amino acid signature (DEWE) that is similar to one found for eukaryotic heat shock proteins: acidic-acidic-hydrophobic-acidic, commonly DDVD (26). There is also evidence that the *Bacteroides nodosus clpB* gene is a heat shock gene: a prokaryotic σ^{32} heat shock promoter consensus sequence is located upstream of the amino acid-coding sequence (42). We think that the fragmentary *Streptococcus mutans* sequence is a ClpB on the basis of N2 and T sequence comparisons, but it is not known if this sequence is also a heat shock protein (42).

The observation that mammalian hsp110 and *E. coli* ClpB both react with yeast hsp104 antibodies suggests that hsp110 proteins also belong to the Clp family (37). Work with hsp110 proteins has been summarized in a recent review (26). Both constitutive and heat-induced 100-kDa proteins concentrate in the nucleolus and are probably associated with the rRNA or proteins that bind rRNA, since hsp110 is released by RNase treatment. It has been suggested that hsp110 protects sensitive ribosome production from temperature shock. Yeast hsp104 is found throughout the nucleus and is also present in the cytosol (27). The suggested role of the eukaryotic hsp110 in the protection of ribosome synthesis may have a prokaryotic analogy. The *clpB* gene immediately precedes the *rrnG* operon on the *E. coli* chromosome (41). At high temperatures, physical proximity of the two genes could ensure local concentration of ClpB for the protection of ribosomes being assembled from the *rrnG* operon.

MOLECULAR CHAPERONES

The heat shock proteins are properly regarded as one of several subclasses of chaperon proteins in the cell (7, 16). The Clp family possesses other chaperonelike attributes as well: universality, a high degree of conservation, high concentration in the cell, and multiple forms or subfamilies within a single cell. Because the functions performed by chaperone proteins are so important to the cell, subfamilies of isoenzymes have been specially tailored to function under different stress conditions and in different physical compartments of the cell.

Molecular chaperones, in addition to coping with stress conditions, protect transitory nonnative proteins as they are being made and when they are unfolded for transport across membranes. Stated most generally, the function of chaperone proteins is to either preserve functional proteins or to break down denatured proteins that have become toxic to the cell. To fulfill their dual functions, either chaperones must possess intrinsic protease activity or they must be associated with a separate protease just as ClpA is associated with ClpP. An intriguing observation that may indicate a broader association of ClpP with chaperone proteins is that *clpP* is preceded on the *E. coli* chromosome by *tig*, the gene for trigger factor, a chaperone protein (25) that is involved in protein transport and plays a role in the regulation of cell division (15). *tig* and *clpP* are separated by approximately 250 bp which contain a Rho-independent terminator for *tig* and promoters for *clpP*. This suggests that the two genes are under independent control, but they might be coexpressed under certain conditions.

Recent studies emphasize that some molecular chaperones not only prevent protein denaturation but also play an active role in the renaturation of denatured and even aggregated proteins (8, 12, 40). DnaK, DnaJ, and GrpE proteins are required for the in vivo reactivation of heat-denatured λ*c*I857 repressor protein (8). The observation that overexpression of hsp70 (DnaK) largely reverses the temperature sensitivity of an hsp104 mutation in *S. cerevisiae* (37) suggests that the function of hsp104 might overlap with that of hsp70. Such overlapping functions might be the reason why Clp null mutations have weak phenotypes. Null mutations of *clpA* cause no readily apparent phenotype (20), and phenotypes caused by *E. coli clpB* mutations are revealed only under rigorously controlled temperature conditions (41).

Viewed as molecular chaperones, the ClpB group is undeniably a stress protein subfamily, while the ClpA group resembles the eukaryotic heat shock cognate (hsc) proteins, which are made constitutively at normal growth temperatures (26). We noted above that antibodies to yeast hsp104 (ClpB) react with several constitutive proteins in Chinese hamster ovary cell extracts (37); therefore, separate Clp protein variants are probably made at normal temperatures in these cells. The plant ClpC examples possess a transit sequence (22) that has been shown to target this protein to the chloroplast in the pea (33), and thus they represent a variant that is targeted to a specific physical compartment of the cell. Whether the ClpC subfamily is functionally distinct from the other Clp subfamilies remains to be answered. The presence of a ClpC in the prokaryotic *M. leprae*, however, suggests that the ClpC subfamily has an ancient origin and is most probably a functionally distinct group. Perhaps ClpC proteins have been tailored to meet some other common stress condition, for example, cold.

CONCLUSIONS

There is no clear answer yet to the question: what do Clp proteins really do? In the preceding sections we have presented some experiments and ideas that should give the reader a better understanding of the functions that Clp proteins might perform. While there is little doubt that some members of the Clp family are involved in proteolysis regulation, the family also has many attributes of molecular chaperones. If Clp proteins are chaperones, some important questions remain to be answered. Can they bind other proteins? Are they associated with the membrane fraction, or are they soluble? Are Clp proteins involved in the transport of other proteins across membranes? Is there additional evidence of their protective role in ribosome assembly? Finally, can Clp proteins renature aberrant proteins? The possibility that Clp proteins may be involved in the process of deciding whether to fix or destroy aberrant proteins is especially exciting. Evidence supporting this idea, which is an important part of the chaperone hypothesis, might resolve more precisely how ClpP protease is associated with the Clp story.

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