A Subfamily of P-Type ATPases with Aminophospholipid Transporting Activity

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The appearance of phosphatidylserine on the surface of animal cells triggers phagocytosis and blood coagulation. Normally, phosphatidylserine is confined to the inner leaflet of the plasma membrane by an aminophospholipid translocase, which has now been cloned and sequenced. The bovine enzyme is a member of a previously unrecognized subfamily of P-type adenosine triphosphatases (ATPases) that may have diverged from the primordial enzyme before the separation of the known families of ion-translocating ATPases. Studies in *Saccharomyces cerevisiae* suggest that aminophospholipid translocation is a general function of members of this family.

The phospholipids of the animal cell plasma membrane are not randomly distributed across the bilayer; the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine, are concentrated in the inner leaflet, and the choline phospholipids, phosphatidylcholine (PC) and sphingomyelin, are concentrated in the outer leaflet (*1*). The loss of this asymmetric distribution and the consequent appearance of PS on the cell surface produces an active surface for assembly of the protease complexes of blood coagulation (*1*), targets blood cells for recognition by the reticuloendothelial system (*2*), and serves as a signal for phagocytosis of apoptotic lymphocytes and neutrophils by macrophages (*3*). PS is normally confined to the cytosolic leaflet of the membrane bilayer by an adenosine triphosphate (ATP)–dependent aminophospholipid translocase identified in plasma membranes (*4*) and in the membranes of secretory vesicles (*5*) and synaptosomes (*6*). The enzyme, a 115-kD Mg^{2+}-dependent ATPase, has been purified from erythrocytes (*7*), chromaffin granules (*8*), and synaptic vesicles (*9*). We have cloned the gene that codes for the ATPase of bovine chromaffin granules (ATPase II). Sequence analysis indicates that it is a member of an ancient and previously unrecognized subfamily of P-type ATPases that includes the product of the *DRS2* gene in yeast (*10*) and ATPases of unknown function cloned from *Plasmodium falciparum* (*11*) and *Caenorhabditis elegans* (*12*). A *drs2* null mutant is defective in PS transport, which suggests that these enzymes are members of a subfamily of ATP-dependent lipid transporters.

ATPase II was purified from bovine adrenal chromaffin granules (*8*) through the second glycerol gradient step. At this stage, ATPase II constituted the major protein in the preparation and was the only polypeptide of its size, as judged by two-dimensional gel electrophoresis. Protein that eluted from a preparative SDS gel and was treated with trypsin yielded fragments from which sequences of 6 and 37 amino acids were obtained. A 78-nucleotide fragment that coded for part of the longer sequence was obtained by a "hemi nesting" polymerase chain reaction (PCR) (*13*) with degenerate oligonucleotides and template DNA from a randomly primed bovine adrenal medulla cDNA library in λgt11 (Clontech). An oligonucleotide from this sequence was used to identify a clone from the library that contained a 1.7-kb insert with an open reading frame (ORF) of 542 amino acids, including the larger amino acid sequence derived from the tryptic peptides. Oligonucleotide sequences derived from this first clone were used to identify clones containing additional contiguous sequence. A second clone with a 1.3-kb insert, including 0.8 kb of new sequence, contained the COOH-terminus of the protein, which proved to be the hexapeptide sequence of the smaller tryptic peptide. A third clone with a 1.6-kb insert extending upstream from the first clone contained an ORF bounded at its 5′ end by stop codons in all frames without a neighboring methionine. Because no other clones from this region were present in the Clontech library, an oligo(dT)-primed calf thymus cDNA library in λgt11 was used as a PCR template with a vector primer and a primer from the 5′ region of the third clone. A 400-nucleotide product was obtained that overlapped with the third clone and contained an ORF ending near two methionines.

From the sequences of the three clones and the PCR product, the sequence of the entire bovine ATPase gene was constructed as an ORF of 1148 amino acids (Fig. 1). Hydrophobicity analysis (*14*) indicated the presence of 10 transmembrane helices with large extramembrane domains between the first and second pairs and between the second and third pairs of transmembrane domains, reminiscent of the canonical structure of the P-type ion transporters. Three consensus sequences (Fig. 2A) are diagnostic of members of this family (*15*): Sequence 1 is implicated in the coupling of ATP hydrolysis to transport, sequence 2 includes the aspartate phosphorylated in the enzyme intermediate, and sequence 3 may participate in ATP binding. All three of these diagnostic sequences are present in the ATPase II, although with different amino acids in several of the variable positions and with an isoleucine replacing a normally invariant leucine near the end of the putative ATP-binding domain. Other less well conserved P-type ATPase consensus sequences (*15*) are also present in the ATPase II (Figs. 1 and 2C), thus confirming the enzyme as a member of this family. The NH_2- and COOH-terminal ends of P-type ATPases, as well as the two

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MPTMRRTVSE IRSRAEGYEK TDDVSEKTSL ADQEEIRTIF INQPQLTKFC NNHVSTAKYN IITFLPRFLY    70
SQFRRAANSF FLFIALLQQI PDVSPTGRYT TLVPLLFILA VAAIKEIIED IKRHKADNAV NKKQTQVLRN   140
GAWEIVHWEK VNVGDIVIIK GKEYIPADTV LLSSSEPQAM CYIETSNLDG ETNLKIRQGL PATSDIKDID   210
SLMRLSGRIE CESPNRHLYD FVGNIRLDGR STVPLGADQI LLRGAQLRNT QWVHGIVVYT GHDTKLMQNS   280
TSPPLKLSNV ERITNVQILI LFCILIAMSL VCSVGSAIWN RRHSGRDWYL NLNYGGANNF GLNFLTFIIL   350
FNNLIPISLL VTLEVVKFTQ AYFINWDLDM HYEPTDTAAM ARTSNLNVEL GQVKYIFSDK TGTLTCNVMQ   420
FKKCTIAGVA YGQNSQFGDE KTFSDSSLLE NLQNNHPTAP IICEFLTMMA VCHTEYQSGK VIRYYQAASP   490
DEGALVRAAK QLNFVFTGRT PDSVIIDSLG QEERYELLNV LEFTSARKRM SVIVRTPSGK LRLYCKGADT   560
VIYDRLAETS KYKEITLKHL EQFATEGLRT LCFAVAEISE SDFQEWRAVY HRASTSVQNR LLKLEESYEL   630
IEKNLQLLGA TAIEDKLQDQ VPETIETLMK ADIKIWILTG DKQETAINIG HSCKLRRKNM GMIVINEGSL   700
DGTRETLSRH CTTLGDALRK ENDFALIIDG KTLKYALTFG VRQYFLDLAL SCKAVICCRV SPLQKSEVVE   770
MVKKQVKVIT LAIGDGANDV SMIQTAHVGV GISGNEGLQA ANSSDYSIAQ FKYLKNLLMV HGAWNYNRGS   840
KCILYCFYKN IVLYIIEIWF AFVNGFSGQI LFERWCIGLY NVMFTAMPPL TLGIFERSCR KEYMLKYPEL   910
YKTSQNALDF NTKVFWVHCL NGLFHSVILF WFPLKALQYG TVFENGRLLI TCCWETFVYT FVVITVCLKA   980
GLETSYWTWF SHIAIWGSIA LWVVFFGIYS SLWPAVPMAP DMSGEAAMLF SSGVFWMGLL FIPVASLLLD  1050
VVYKVIKRTA FKTLVDEVQE LEAKSQDPGA VVLGKSLTER AQLLKNVFKK NHVNLYRSES LQQNLLHGYA  1120
FSQDENGIVS QSEVIRAYDT TKQRPDEW                                                 1148
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Fig. 1. Predicted amino acid sequence of bovine ATPase II (*19*). P-type ATPase consensus sequences are shown in boxes. Transmembrane helices are underlined. The 37–amino acid sequence and the hexapeptide sequence obtained from tryptic peptides are double-underlined. The nucleotide sequence has been deposited in GenBank (accession number U5110).

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large extramembrane domains, are oriented on the cytosolic side of the membrane (*15*), which implies that the extracytosolic domains of the ATPase II (between transmembrane helices 1–2, 3–4, 5–6, 7–8, and 9–10) are very small, with the largest one (between transmembrane helices 3 and 4) no more than ~20 amino acids in length. No glycosylation motifs are present in any of these regions; this finding is consistent with the close correspondence between the apparent molecular size of the ATPase estimated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (~120 kD as isolated from chromaffin granules) and the molecular size calculated from the derived sequence (130 kD).

A search of the nonredundant group of databases at the National Library of Medicine indicated little similarity between the ATPase II and multidrug resistance (MDR) proteins, kinesins, or the 115-kD subunit of V-ATPases (including the ATPase I from chromaffin granules). The ATPase II is weakly similar to various Ca^{2+}-dependent ATPases and is even less similar to Na^+- and K^+-dependent ATPases. However, the sequence is very similar to that of the predicted protein products of the *DRS2* gene of yeast *S. cerevisiae* (*10*), the *pfATPase2* gene of *P. falciparum* (*11*), and the CELT24H7.5 locus of *C. elegans* (*12*). Reanalysis of the hydrophobicity profile of the DRS2 sequence (*14*) and comparison with the ATPase II revealed three membrane-spanning domains in addition to the seven previously reported (*10*). Ten transmembrane domains were also found in the *P. falciparum* sequence. The reported *C. elegans* sequence contains only eight domains and lacks the critical transmembrane helices separating the two large cytosolic domains. However, if introns in the genomic sequence (National Center for Biotechnology Information gi:861319, GenBank accession number U28940) are moved to positions 9339 to 9462 and 9574 to 10,126, the new ORF contains the missing transmembrane domains (Fig. 2C). The bovine protein sequence is most similar to the yeast sequence (47% identity and 67% similarity) (Fig. 2B).

A more detailed analysis of the four genes suggests that they constitute a subfamily of P-type ATPases distinct from the metal ion transporters that constitute the bulk of this class of enzymes. In the positions where the ATPase II–related genes differ from the consensus sequences previously determined for this family (*15*), all four often share the same amino acids, for example, in positions 1, 2, and 5 of sequence 1 and position 10 of sequence 3. Moreover, in sequence 2, the amino acid two positions upstream from the phosphorylated aspartate—a position occupied by cysteine, alanine, methionine, or leucine in the 61 proteins considered by Fagan and Saier (*15*)—is a phenylalanine in three members of the group. These comparisons indicate that this group of proteins forms a distinct subfamily of transporting ATPases, which may have diverged from the primordial P-type ATPase gene before the duplications that gave rise to the major ion-translocating ATPase families. A similar conclusion is suggested by a phylogenetic analysis of the sequences.

There is no definitive evidence that yeasts are capable of aminophospholipid-specific transport [however, see (*16*)]. Accordingly, we labeled the outer leaflet of the plasma membrane of yeast with fluorescent 2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]hexanoyl (NBD)–PS and measured the probe remaining in this location by extraction into buffer containing bovine serum albumin (BSA). NBD-PS was rapidly transported from the yeast surface (Fig. 3A), but NBD-PC was not transported (Fig. 3B). The specificity of transport and assay conditions that block endocytosis (*16*) eliminated this mechanism for uptake. PS transport was abolished in a *drs2* deletion mutant (Fig. 3A), which suggested that

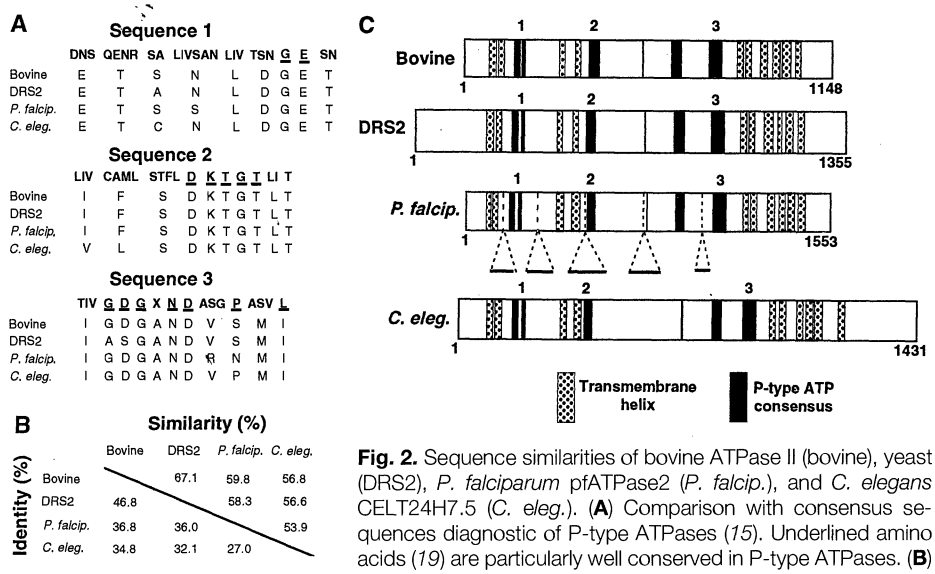

Fig. 2. Sequence similarities of bovine ATPase II (bovine), yeast (DRS2), *P. falciparum* pfATPase2 (*P. falcip.*), and *C. elegans* CELT24H7.5 (*C. eleg.*). (**A**) Comparison with consensus sequences diagnostic of P-type ATPases (*15*). Underlined amino acids (*19*) are particularly well conserved in P-type ATPases. (**B**) Paired comparison of sequences, obtained with the gap procedure of the GCG sequence analysis package (*20*). (**C**) Diagrammatic comparison of the complete protein sequences. Numbers above each diagram refer to the three diagnostic consensus sequences in (A). Inserts characteristic of the *P. falciparum* parasite (*11*) have been extracted and their positions are indicated by dashed lines.

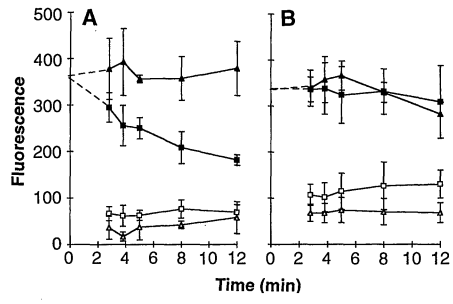

Fig. 3. Transport of phospholipid analogs by wild-type and *drs2* yeast strains. The outer leaflet of the plasma membrane of yeast was labeled with fluorescent phospholipid analogs, and internalization was measured (*21*). Times are from addition of probe; curves have been extrapolated back to initial labeling (dashed line). Values are mean ± SE for three separate experiments (each with duplicate samples). NBD-PS transport (**A**) and NBD-PC transport (**B**) are shown for wild-type (■) and DRS2 null (▲) strains; background supernatant values (no BSA added) are also shown for wild-type (□) and DRS2 null (△) strains.

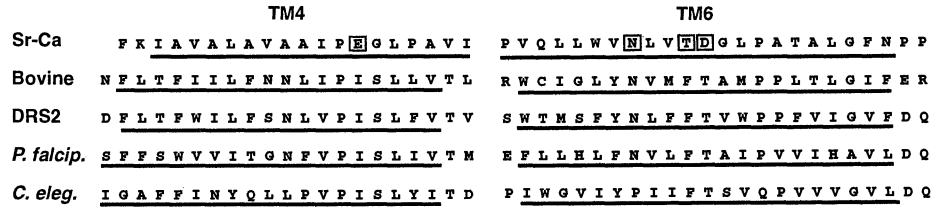

Fig. 4. Comparison of transmembrane domains 4 and 6 (TM4 and TM6) of the Ca^{2+} transporter from chicken sarcoplasmic reticulum (Sr-Ca) with the corresponding bovine, yeast (DRS2), *P. falciparum*, and *C. elegans* sequences (*19*). Sequences within transmembrane helices are underlined. Amino acids important to Ca^{2+} binding (*17*) are shown in boxes.

transbilayer transport of lipids is the function common to this ATPase subfamily.

Although PS is a charged molecule, its amphipathic character distinguishes it from the simple ions transported by most P-type ATPases. Several glutamates as well as asparagine and threonine buried within transmembrane helices 4, 5, 6, and 8 are important to cation transport, particularly by the Ca^{2+}-dependent ATPases (*17*). Tentative sequence alignment indicates that these positions in transmembrane helices 4 and 6 of the ATPase II subfamily are replaced by amino acids with bulky hydrophobic side chains, which might interact with the hydrophobic portions of the amphipathic phospholipid molecules (Fig. 4).

Down-regulation of the aminophospholipid translocase activity is an early step in apoptosis (programmed cell death) in lymphocytes (*18*), a step that contributes to the appearance of PS on the cell surface as a recognition signal for phagocytosis. Identification of a homolog in *C. elegans* may aid in elucidating whether loss of lipid asymmetry plays a role in recognition of apoptotic cells during development in this organism.

REFERENCES AND NOTES

1. A. J. Schroit and R. F. A. Zwaal, *Biochim. Biophys. Acta* **1071**, 313 (1991); P. Williamson and R. A. Schlegel, *Mol. Membr. Biol.* **11**, 199 (1994).
2. L. McEvoy, P. Williamson, R. A. Schlegel, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3311 (1986); T. M. Allen, P. Williamson, R. A. Schlegel, *ibid.* **85**, 8067 (1988); D. Pradhan, P. Williamson, R. A. Schlegel, *Mol. Membr. Biol.* **11**, 181 (1994).
3. V. A. Fadok *et al.*, *J. Immunol.* **148**, 2207 (1992); V. A. Fadok *et al.*, *ibid.* **149**, 4029 (1992); R. A. Schlegel, M. Callahan, S. Krahling, P. Williamson, in *Proceedings of the Sixth International Conference on Lymphocyte Activation and Immune Regulation: Cell Cycle and Programmed Cell Death in the Immune System*, S. Gupta and J. J. Cohen, Eds. (Plenum, New York, in press).
4. M. Seigneuret and P. F. Devaux, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3751 (1984); A. Zachowski, A. Herrmann, A. Paraf, P. F. Devaux, *Biochim. Biophys. Acta* **897**, 197 (1987); O. C. Martin and R. E. Pagano, *J. Biol. Chem.* **262**, 5890 (1987); A. Bogdanov, B. Verhoven, R. A. Schlegel, P. Williamson, *Biochem. Soc. Trans.* **21**, 271 (1993).
5. A. Zachowski, J. P. Henry, P. F. Devaux, *Nature* **340**, 75 (1989).
6. A. Zachowski and Y. Morot Gaudry-Talarmain, *J. Neurochem.* **55**, 1352 (1990).
7. G. Morrot, A. Zachowski, P. F. Devaux, *FEBS Lett.* **266**, 29 (1990); M. E. Auland, B. D. Roufogalis, P. F. Devaux, A. Zachowski, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10938 (1994).
8. Y. Moriyama and N. Nelson, *J. Biol. Chem.* **263**, 8521 (1988).
9. B. W. Hicks and S. M. Parsons, *J. Neurochem.* **58**, 1211 (1992); X. Xie, D. K. Stone, E. Racker, *J. Biol. Chem.* **264**, 1710 (1989).
10. T. L. Ripmaster, G. P. Vaughn, J. L. Woolford, *Mol. Cell. Biol.* **13**, 7901 (1993).
11. S. Krishna, G. M. Cowan, K. J. Robson, J. C. Meade, *Exp. Parasitol.* **78**, 113 (1994); F. Trottein and A. Cowman, *Eur. J. Biochem.* **227**, 214 (1995).
12. R. Wilson *et al.*, *Nature* **368**, 32 (1994).
13. H. Li, X. Cui, N. Arnheim, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4580 (1990).
14. D. Eisenberg, E. Swarz, M. Kamaromy, R. Wall, *J. Mol. Biol.* **179**, 125 (1984).
15. M. J. Fagan and M. H. Saier Jr., *J. Mol. Evol.* **38**, 57 (1994).
16. L. S. Kean, R. S. Fuller, J. W. Nichols, *J. Cell Biol.* **123**, 1403 (1993).
17. D. M. Clarke, T. W. Loo, G. Inesi, D. H. MacLennan, *Nature* **339**, 476 (1989).
18. B. Verhoven, R. A. Schlegel, P. Williamson, *J. Exp. Med.* **182**, 1597 (1995).
19. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
20. S. B. Needleman and C. D. Wunsch, *J. Mol. Biol.* **48**, 443 (1970).
21. Wild-type *S. cerevisiae* (DS94) and a *drs2* null allele strain (JWY2197) were grown to mid–log phase in yeast extract–peptone-dextrose medium. Cells were washed twice with yeast extract–peptone-sorbitol medium containing 20 mM sodium azide, resuspended in the same medium, and shaken at 30°C for 45 min to inhibit endocytosis (*16*). After one wash with ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) containing 1 mM phenylmethylsulfonyl fluoride and two washes with PBSSA (PBS containing 10 mM sorbitol and 20 mM sodium azide) at 4°C, the cells were resuspended at 6×10^8 cells/ml in the same buffer and incubated on ice for 1 hour. Either NBD-PS or NBD-PC (1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphoserine or -phosphocholine; Avanti Polar Lipids) was added to a final concentration of 1 mM from a stock solution in dimethylsulfoxide. After 1 min on ice, the cells were centrifuged; the supernatant was discarded and the cells were resuspended in PBSSA. Immediately upon resuspension and at 1, 2, 5, and 9 min thereafter, four portions (50 μl) were withdrawn, two of which were each mixed with 5 μl of fatty acid–free BSA (Sigma, 10% w/v) in PBSSA, and two of which were mixed with 5 μl of PBSSA alone. After 1 min on ice, the cells were sedimented and 45 μl of the supernatant was transferred to 1500 μl of 1% Triton X-100 in water. Fluorescence intensity was measured at 530 nm during excitation at 470 nm.

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Principles of Chaperone-Assisted Protein Folding: Differences Between in Vitro and in Vivo Mechanisms

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Molecular chaperones in the eukaryotic cytosol were shown to interact differently with chemically denatured proteins and their newly translated counterparts. During refolding from denaturant, actin partitioned freely between 70-kilodalton heat shock protein, the bulk cytosol, and the chaperonin TCP1–ring complex. In contrast, during cell-free translation, the chaperones were recruited to the elongating polypeptide and protected it from exposure to the bulk cytosol during folding. Posttranslational cycling between chaperone-bound and free states was observed with subunits of oligomeric proteins and with aberrant polypeptides; this cycling allowed the subunits to assemble and the aberrant polypeptides to be degraded. Thus, folding, oligomerization, and degradation are linked hierarchically to ensure the correct fate of newly synthesized polypeptides.

Although the amino acid sequence of a polypeptide chain contains the information that determines the three-dimensional structure of the functional protein (*1*), the folding of many proteins in vivo requires the assistance of a preexistent machinery of molecular chaperone proteins (*2*). These components prevent off-pathway folding reactions that lead to aggregation. Two functionally distinct chaperone families, the 70-kD heat shock proteins (Hsp70s) and the chaperonins, have been implicated in protein folding in the cytosol. The Hsp70s bind to extended hydrophobic peptide segments in an adenosine triphosphate (ATP)–dependent manner that requires the cooperation of members of the DnaJ/Hsp40 family of chaperones (*3*). The chaperonins are large cylindrical complexes consisting of two stacked rings of seven to nine subunits each. Partially folded polypeptides bind within the chaperonin central cavity and reach the native state through multiple, ATP hydrolysis–dependent cycles of binding and release. The bacterial chaperonin GroEL is homo-oligomeric, whereas the eukaryotic chaperonin TCP1–ring complex (TRiC) is composed of eight different but homologous subunits (*4–6*).

Molecular chaperones are thought to have a general role in the folding of newly synthesized polypeptides in vivo (*7–9*). However, our present understanding of chaperone mechanisms relies predominantly on the results of in vitro studies, in which a complete polypeptide, unfolded by chem-

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