Molecular Cloning and Characterization of a Mouse Homolog of Bacterial ClpX, a Novel Mammalian Class II Member of the Hsp100/Clp Chaperone Family*

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In this paper, we present the molecular cloning and characterization of a murine homolog of the *Escherichia coli* chaperone ClpX. Murine ClpX shares 38% amino acid sequence identity with the *E. coli* homolog and is a novel member of the Hsp100/Clp family of molecular chaperones. ClpX localizes to human chromosome 15q22.2–22.3 and in mouse is expressed tissue-specifically as one transcript of ~2.9 kilobases (kb) predominantly within the liver and as two isoforms of ~2.6 and ~2.9 kb within the testes. Purified recombinant ClpX displays intrinsic ATPase activity, with a *K_m* of ~25 μM and a *V_max* of ~680 pmol min⁻¹ μg⁻¹, which is active over a broad range of pH, temperature, ethanol, and salt parameters. Substitution of lysine 300 with alanine in the ATPase domain P-loop abolishes both ATP hydrolysis and binding. Recombinant ClpX can also interact with its putative partner protease subunit ClpP in overexpression experiments in 293T cells. Subcellular studies by confocal laser scanning microscopy localized murine ClpX green fluorescent protein fusions to the mitochondria. Deletion of the N-terminal mitochondrial targeting sequence abolished mitochondrial compartmentalization. Our results thus suggest that murine ClpX acts as a tissue-specific mammalian mitochondrial chaperone that may play a role in mitochondrial protein homeostasis.

The Hsp100/Clp family of ATPases constitutes a group of molecular chaperones that participate in a broad range of biological processes in both prokaryotes and eukaryotes. Sequence similarity and conservation of structural features among the over 70 known family members define two classes, which are further subdivided into eight subfamilies (1). All Hsp100/Clps examined have been demonstrated to assemble into homooligomeric ring-shaped structures and to modulate substrates in an ATP-dependent manner (1, 2). Specific substrate recognition occurs through protein-protein interactions directed by the PDZ-like domains of the Hsp100/Clp family members (3). Members of the family participate in the disaggregation of improperly folded and damaged proteins, the facilitation of DNA transposition, the selective coordination of substrate degradation, the regulation of the inheritance of prion-like factors, and the modulation of gene expression (reviewed by Schirmer *et al.* (1)). Despite the involvement of Hsp100s in such diverse processes, it is the conserved structural organization of the members that suggests that these varied functions may involve a common mechanism governing disassembly of high-order quartenary protein complexes (1, 4–6).

*E. coli* ClpX is a heat-shock protein (7–9) of the class II Hsp100/Clp subfamily (1) and can act alone as a molecular chaperone. It is an essential component of the Mu transposase life cycle where it mediates dissociation of stable MuA tetramer-DNA complexes (10, 11). Deletion of ClpX blocks the growth of Mu by arresting transposition at the transition between the recombination and DNA replication stages (10–12). The molecular chaperone properties of *E. coli* ClpX are further supported by its capacity to prevent the heat inactivation of the bacteriophage λO replication protein, to dissociate preformed λO aggregates, and to stimulate the binding of λO to oriA DNA (13).

While *E. coli* ClpX can function alone as a *bona fide* molecular chaperone, it also contributes to a number of processes as a regulatory subunit of the broad specificity, energy-dependent protease ClpP. In *E. coli*, ClpX and ClpP are translated from a single heat-shock-inducible transcript in accordance with their involvement in stress tolerance (7–9). In this two-component chaperone-protease system, ClpX does not refold proteins to mediate functional reactivation but rather utilizes its chaperone activity to selectively target specific substrates for degradation by channeling them into the proteolytic chamber of the two-ring ClpP tetradecamer. Negative staining electron microscopy (14) and crystallographic analysis of ClpP (15) reveal a structural organization that is homologous to that of the eukaryotic 26 S proteasome. Specific targets of ClpXP include λO (7, 16), starvation sigma factor (σ⁵) (17), SsrA-tagged proteins generated from defective mRNAs (18), the Ph protein of plasmid prophage P1 (19), the Caulobacter crescentus cell cycle regulator CtrA protein (20, 21), MuA (22), and the Mu repressor protein (23, 24). Many of these substrates possess 7–11 amino acid C-terminal peptides that are required for recogni-
Protein homeostasis.

While prokaryotes possess pan-cellular chaperone distribution, eukaryotes require compartment-specific chaperones to negotiate and maintain polypeptide chains within the appropriate tertiary structure and to facilitate the degradation of unsalvageable or transient protein molecules and complexes (25, 26). As expected from the proposed endosymbiotic origins of the mitochondria (27, 28), the molecular chaperones that direct mitochondrial function display significant sequence homology to bacterial counterparts. In particular, mitochondrial proteins Hsp70, chaperonins Hsp60/Hsp10 and Hsp78 are homologous to _E. coli_ DnaK, GroEL/GroES, and the class I Hsp100/Ctp family, respectively. The conspicuous absence of an eukaryotic homolog of the ClpX class II Hsp/Ctp subfamily was remedied following the sequencing of the complete genome of _Saccharomyces cerevisiae_ (29, 56) and the subsequent demonstration that the gene product termed MexClp1p partitioned to the mitochondria (30). However, a mammalian homolog had remained until this point unidentified. The existence of such a member has been strongly implied by the cloning of a human homolog of ClpP that sorts to the mitochondrial matrix (31, 32).

In this report, we describe the identification and initial characterization of murine ClpX, a novel mammalian member of the Hsp100/Ctp family of molecular chaperones that displays distinct sequence similarity with its _E. coli_ counterpart. We demonstrate that murine ClpX is directed to the mitochondria by an N-terminal targeting peptide. In line with its likely role as a mitochondrial molecular chaperone, we show that ClpX possesses an intrinsic ATPase activity that is resilient in _vitro_ to fluctuations in reaction conditions reflecting environmental stress. Its capacity to interact with mouse ClpP in mammalian overexpression experiments suggests that mouse ClpXP may represent a novel system for the regulation of mitochondrial protein homeostasis.

**EXPERIMENTAL PROCEDURES**

**Cloning of the Murine Homolog of Bacterial ClpX, Chromosomal Localization, and Tissue Expression—** Blast searches of the data base of expressed sequence tags (dBEST) (33) using _E. coli_ ClpX revealed the partial murine ClpX EST clone A0135832 (Image Consortium clone 441677). This ClpX fragment was random prime (\(\alpha\)-\(\alpha\)-\(P\)\)dCTP body-labeled PCR \(1\) fragment generated using primers 467 (5'-ATGTTAGGAAGACTGGGGACG-3') and 238 (5'-TATACACGGC-3') and the subsequent cloning of both strands (Utah State University Biotechnology Center). A human chromosomal location of ClpX was assigned by analyzing the physical map of the human genome available at http://www.ncbi.nlm.nih.gov/genemap/ (35, 36). A mouse Multiple Tissue Northern (CLON-TECH) was hybridized as recommended by the manufacturer with an \(\alpha\)-\(\alpha\)-\(P\)\)dCTP body-labeled PCR \(2\) fragment generated using primers 487 (5'-ATGTTAGGAAGACTGGGGACG-3') and 238 (5'-TATACACGGC-3'). The C terminus of proteins were considered homologous to the C-terminal targeting sequence of bacterial ClpX substrates if all four of the following criteria were fulfilled: 1) the final two amino acids were nonpolar, 2) the preceding 7 amino acids were preferentially polar or charged, 3) at least 6 of the 11 amino acids were similar to the final amino acids of the _SorA_ tag (18), and 4) at least 1 of the first 2 amino acids was nonpolar.

**Construction of ClpX in Vitro Transcription-Translation and Mammalian Expression Vectors—** To create serial N-terminal deletions of ClpX in the Sp6 promoter driven vector pCE27, PCR products bearing optimal Kozak translation start sequences were cloned between the BamHI of the polylinker and an Aval site internal to ClpX (pCE27ClpXM, pCE27ClpXM2, pCE27ClpXM3, pCE27ClpXα65). To generate fusions to the C terminus of glutathione S-transferase of the N-terminal serial deletions, BamHI fragments from the start ATG to the 3'-untranslated region (nt 2439) were subcloned from the pCE27 vectors into the eIF2 promoter vector pEBB (pEBBClpa53 3'-HA). All PCR-generated constructs were confirmed by sequencing.

**Cell Culture and Recombinant Eukaryotic Protein Expression—** The human embryonic kidney fibroblast line, 293T, was grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum at 37 °C in a 5% CO_2_ atmosphere. GST fusion proteins of ClpX were overexpressed and purified from transient transfections of 293T cells at 25% confluence using the calcium phosphate precipitation method.

**ATPase and Nucleotide Binding Assays—** Indicated concentrations of recombinant GSTClpX wild-type and mutant proteins were incubated in a 20-μl reaction with 25 μl Tris, pH 7.4, 10 mM MgCl_2_, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin and sonicated exten- sively. The lysate was cleared in a microcentrifuge for 15 min at 14,000 rpm and 4 °C. An aliquot was removed for protein expression evalua- tion, and the remainder of the lysate was incubated on glutathione beads for 2 h at 4 °C with gentle rocking. The beads were washed five times with mild vortexing in immunoprecipitation lysis buffer containing 20 μl Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 μM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM of pepstatin and sonicated exten- sively. The lysate was cleared in a microcentrifuge for 15 min at 14,000 rpm and 4 °C. An aliquot was removed for protein expression evalua- tion, and the remainder of the lysate was incubated on glutathione beads for 2 h at 4 °C with gentle rocking. The beads were washed five times with mild vortexing in immunoprecipitation lysis buffer and finally resuspended in 2 x SDS-PAGE loading buffer. The interaction was subsequently evaluated with Western analysis.

**Cellular Localization of Recombinant ClpX—** Recombinant proteins, GSTClpX full-length or N-terminal truncations in pEBG, and 3'-HA-tagged ClpP in pEBB were transiently overexpressed in mouse keratinocytes and processed as described previously (38) and dialyzed in 25 mM Tris, pH 8.0, 200 mM NaCl, 2 mM EDTA, 10 mM β-mercaptoethanol, and 20% glycerol. Protein quantita- tion was conducted following SDS-PAGE and Coomassie staining using dilutions of bovine serum albumin as a standard.

**Results**

**ClpX/ClpP Protein Interaction—** Recombinant proteins, GSTClpX full-length or N-terminal truncations in pEBG, and 3'-HA-tagged ClpP in pEBB were transiently overexpressed in 293T cell culture using calcium phosphate precipitation. Cells were harvested 48 h post-transfection and processed as described above. Twenty-four hours post-transfection, the cells were harvested and processed as described above.

**Cellular Localization of Recombinant ClpX—** Recombinant proteins, GSTClpX full-length or N-terminal truncations in pEBG, and 3'-HA-tagged ClpP in pEBB were transiently overexpressed in 293T cells as described above. Thirty-six hours post-transfection, the cells were harvested and processed as described above. Twenty-four hours post-transfection, the cells were harvested and processed as described above.
ing three washes in PBS + 1% BSA, the cells were incubated for 10 min at room temperature with 5 μg/ml 4′-diaminidino-2-phenylindole (Sigma) in PBS + 1% BSA. Following three washes in PBS + 1% BSA, the coverslips were mounted. Images were acquired using confocal laser microscopy.

RESULTS

Isolation of a Murine Homolog of Bacterial ClpX and Evolutionary Conservation of Functional Domains—Due to our laboratory’s interest in the remodeling of higher order nucleoprotein complexes in mammalian transposition and recombination reactions, we screened the data base of expressed sequenced tags (dBEST) (33) for a mouse homolog of the E. coli ATP-dependent remodeling factor ClpX. Identification of EST clone Aa013832 permitted isolation of a full-length cDNA of 2847 base pairs encoding mouse ClpX (Fig. 1A) from a keratinocyte library. The potential open reading frame is demarcated by two stop codons, a 5′ TGA (nt 128–130) and a 3′ TAA (nt 2075–2077), which contain two possible sites of translation initiation, ATG1 (nt 179–181: M1) and ATG2 (nt 287–289: M2). From ATG1, the cDNA comprises an open reading frame of 1896 base pairs and encodes a predicted protein of 632 amino acids with a molecular mass of 69,174 Da and a theoretical isoelectric point of 7.76. The mouse ClpX as compared with Caenorhabditis elegans, E. coli, and S. cerevisiae ClpX demonstrates 40, 38, and 32% amino acid identity and 59, 46, and 48% similarity, respectively (Fig. 1B). A consensus polyadenylation site (AATAAA) is located 14 base pairs before the 3′ poly(A) start (Fig. 1A).

Functional analysis of mouse ClpX is dependent upon an understanding of its component motifs. We thereby note that the primary structure of the 69-kDa mouse ClpX polypeptide displays three discernable domains that are evolutionary conserved (Fig. 1A). The cDNA and protein sequences of murine ClpX are displayed. Putative translation initiation and stop codons are emphasized in uppercase letters. Two possible start methionines are indicated (M1 and M2) in bold and the three consensus cleavage sites (I1 of the R-2 motif and I2 and I3 of the R-10 motif) (42) for the matrix processing peptidase are marked. The predicted Ca2+-zinc finger is underlined by dots with the putative Zn2+ coordinating cysteines highlighted in bold. The P-loop and Walker B sites of the ATPase motif are single underlined with lysine 300 of the GKT motif indicated in bold. The region displaying homology to PDZ domains is double underlined with the boundary between PDZ domain A and B demarcated by the GKT motif. The polyadenylation consensus is single underlined and in small caps.
served through *E. coli* (Fig. 1, A and B). First, along with the C. elegans and *E. coli* homologs, murine ClpX possesses toward the N terminus a C4-type zinc finger (amino acids 106–131) of unknown function. Second, ClpX bears a characteristic ATPase motif with a classic P-loop (amino acids 290–305) and Walker B Mg\(^{2+}\) binding pocket (amino acids 355–362) with distinct similarity to those of the F$_1$-ATPase and P-type transporters (40). Third, the C terminus contains two tandem PDZ-like domains, which display 44, 31, and 41% identity and 65, 58, and 58% similarity with the PDZ-like domains of *C. elegans*, *S. cerevisiae*, and *E. coli*, respectively. In *E. coli*, these domains mediate specific substrate recognition (3). In addition to these three highly conserved motifs, mouse ClpX possesses at the N terminus an apparent mitochondrial targeting sequence (41–43) spanning as much as the first 65 amino acids of the protein (Fig. 1A). This region is characterized by the predicted capacity to form an amphiphilic helix coupled with a biased distribution of positively charged amino acids (7 arginines), an abundance of hydroxylated residues (13 serines or threonines), and a paucity of negatively charged amino acids (1 aspartate and 1 glutamate). Maturation of the ClpX preprotein can be predicted to result following cleavage by the mitochondrial processing peptidase at three putative sites, one site within an R-2 motif and another two sites within an R-10 motif (Fig. 1A) (42, 43).

To determine whether translation preferentially initiates at AUG 1 or 2, the migration was compared between full-length ClpX cDNA in *vitro* translation products and the products from truncated forms of ClpX starting from either ATG1 (ClpXM1), ATG2 (ClpXM2), or an N-terminal deletion of the first 65 amino acids (ClpXΔ65) (Fig. 2B). While over 98% of the translation products from the full-length cDNA (Fig. 2B, arrow a in lane 1) co-migrated with ClpXM1 (lane 2), a minor species was produced (arrow b in lane 1) that co-migrated with the ClpXM2 product (lane 3). The preponderance of initiation at AUG1 suggests that this is the biologically relevant start site. However, while translation initiation at AUG2 may be specific to the *in vitro* transcription/translation system and may not occur *in vivo*, the possibility that the two translation isoforms are targeted differentially to separate subcellular compartments lead us to characterize both start forms in subsequent studies.

**Tissue-specific Expression and Assignment of Chromosomal Localization—**Northern blot analysis was used to study the tissue distribution of ClpX encoding mRNA. Samples of poly(A)$^+$ RNA from eight different BALB/c mouse tissues were hybridized with labeled probe specific for ClpX (Fig. 2A). The relative expression level is highly variable between different tissues. ClpX was expressed predominantly in the liver as a single transcript of ~2.9 kb and in the testes as two transcripts of ~2.6 and ~2.9 kb. The length of the 2.9-kb mRNA corresponds to the size of the full-length cDNA (Fig. 1A). The two mRNAs in the testes may result from alternative RNA splicing. Lower expression of the 2.9-kb ClpX transcript was detected in the heart and kidney. Very low levels were observed in skeletal muscle (upon overexposure) with no apparent expression detectable in the brain, spleen, and lung.

Human chromosomal localization of ClpX was assigned using the Gene Map of the Human Genome, which is a physical map of cDNA-based sequence-tagged sites (35, 36) available on the World Wide Web. This resource of currently 30,181 genes utilizes the unique 3'-untranslated region of a cDNA to position ESTs relative to microsatellite markers by radiation hybrid mapping with an error rate of 1.08%. ClpX was typed on a Genebridge4 radiation hybrid panel using PCR primers designed to the 3'-untranslated region of the human gene and determined to map to chromosome 15q22.2–22.3 between fixed reference markers D15S125 and D15S216.
recruit ATP. At 4 °C, wild-type GSTClpXM1, GSTClpXM2, and GSTClpXΔ65 associated with both ATP and ADP (Fig. 4C). The ratio of bound ATP to bound ADP was about 1:5. This association required the presence of Mg2+ (data not shown). In contrast, GSTClpXΔ65K300A binding to ATP at 4 °C was negligible (Fig. 4C), suggesting that Lys 300 is critical for both ATP binding and hydrolysis.

Both of the two previously cloned members of the Hsp100/Clp family in S. cerevisiae, Hsp104 and Hsp78, mediate stress tolerance under conditions of extreme temperature (6, 45), and Hsp104 is also of critical importance for tolerance to ethanol (46). Hence, we analyzed the ATPase profile of ClpX under various conditions reflecting environmental stress (4). Interestingly, ClpX is resilient to varied alterations in reaction conditions. ATP hydrolysis was essentially unaltered over a pH range of 6.8–8.8 (Fig. 5A) and decreased only 15% when the salt range was varied from the physiological value of 150–450 mM NaCl (Fig. 5B). In addition, ClpX retained 75% of wild-type activity in 20% ethanol (Fig. 5C) and 90% of wild-type activity at 55 °C (Fig. 5D). The ability to function across a broad scope of reaction parameters suggests that ClpX may function along with the other Hsp100/Clp family members in response to cellular stress.

**ClpX Interacts with ClpP**—The absence of an open reading frame in the complete yeast genome demonstrating distinct homology to bacterial ClpP (29, 56) suggests the possibility that ClpX may have evolved in eukaryotes to function independently of ClpP. To address whether mouse ClpX could still as-

**TABLE I**

ATPase activity of ClpX in the presence of various divalent cations

| Ion   | ClpX | ClpX pmol Pi released % |
|-------|------|-------------------------|
| Mg2+  | 378 ± 18 | 100                      |
| Mn2+  | 355 ± 25 | 94                      |
| Ca2+  | 34 ± 5   | 9                       |
| Zn2+  | 3 ± 2    | 0                       |
| Fe2+  | 8 ± 2    | 2                       |
| Co2+  | 2 ± 2    | 0                       |
| Cd2+  | 1 ± 2    | 0                       |
| Ni2+  | 2 ± 2    | 0                       |

Novel Mammalian Mitochondrial ClpX Class 2 Hsp100/Clp Member

![Figure 3](image.png)

**Fig. 3. Characterization of the ATPase activity of murine ClpX.** A, SDS-PAGE analysis of GSTClpXM1 (lane 1), GSTClpXM2 (lane 2), and GSTClpXΔ65 (lane 3) purified following transient overexpression in 293T cells. B, picomoles of ATP hydrolyzed versus concentration of ClpX. The amount of ATP hydrolyzed in 6 min at 37 °C by increasing amounts of GSTClpXΔ65 (0.006–0.2 μM) in reactions containing 1000 pmol ATP (50 μM final ATP concentration) was determined. Pi was separated from unhydrolyzed ATP by thin layer chromatography, and amounts were quantitated following autoradiography. C, time course of ATPase activity; pmolmes of ATP hydrolyzed at 37 °C over time (minutes) by 0.05 and 0.025 μM GSTClpXΔ65 with 1000 pmol ATP (50 μM) as the initial substrate amount. D, rate of hydrolysis was determined from the picomoles of Pi released over 6 min by 100 ng of ClpX (0.05 μM). Km and Vmax values were also derived from a Lineweaver-Burk plot of the same values (graph not shown). Reaction rates were calculated using 0.05 μM GSTClpXΔ65 at initial ATP concentration values of 5, 10, 15, 25, 50, 100, 200, and 250 μM ATP. GSTClpXM1 and GSTClpXM2 yielded similar Km and Vmax values.
associate to form a complex with mouse ClpP, we performed co-precipitation assays between N-terminal serial deletions of ClpX fused in frame with the C termi-nus of glutathione S-transferase and ClpP (amino acids 56–272) with a C-terminal HA tag. Recombinant proteins were overexpressed in 293T cells and interaction was evaluated by affinity precipitation of complexes on glutathione beads. GSTClpXM1 does not interact nonspecifically with any anti-HA cross-reactive species in 293T cells not transfected with ClpP3 HA (Fig. 6, lane 1 in the middle panel). GST alone did not interact with ClpP3 HA (Fig. 6, lane 2 in the middle panel). GSTClpXM1, GSTClpXM2, and GSTClpXΔ65 all co-precipitated ClpP3 HA with approximately equal efficiency (Fig. 6, lanes 3–5 in the middle panel). Precipitated levels of the GST and GSTClpX proteins were evaluated by Western analysis (Fig. 6, upper panel). Expression levels of ClpP3 HA were assessed by Western analysis of whole cell extracts from the transfected cells (Fig. 6, lower panel). As a control, GSTClpX proteins did not interact with various HAtagged forms of Rag 1 and 2 proteins (data not shown). In all, the data demonstrate the capacity of mouse ClpX/ClpP to form a stable complex. This interaction suggests that mouse ClpX may function analogously to E. coli ClpX as an energy-dependent regulator of ClpP function.

Subcellular Sorting of Mouse ClpX-Green Fluorescent Protein Fusions—The presence of an apparent mitochondrial tar-

geting sequence on the N terminus of ClpX strongly suggested that ClpX would localize to the mitochondria along with its interacting partner protein ClpP (31). To explore the intracellular distribution of ClpX, we fused the C terminus of the full-length ClpXM1 preprotein with the N terminus of a redshifted variant of green fluorescent protein (EGFP). In addition, the possibility that use of the second ATG (M2) might direct differential compartmentalization of this in vitro minor translation species (Fig. 2B) was explored using a ClpXM1-EGFP fusion initiated directly from the second methionine. The fusions were overexpressed in 293T cells and analyzed using confocal laser scanning microscopy. With EGFP alone, fluorescence was observed throughout the nucleus and cytoplasm (Fig. 7A). On the other hand, both the ClpXM1-EGFP and ClpXM2-EGFP fusions generated fluorescence in the form of discrete cytoplasmic rod-like elements, suggesting distribution of ClpX to the paracrystalline structures of the mitochondria (Fig. 7, B and C). To verify that the punctate staining suggestive of mitochondrial localization was dependent on the integrity of the N-terminal 65-amino acid targeting peptide, we analyzed distribution of ClpXΔ65-EGFP lacking the targeting sequence. Consistent with the existence of an N-terminal mitochondrial targeting peptide, this deletion ablated the punctate fluorescence and, in turn, generated homogenous cytoplasmic staining (Fig. 7D). Subcellular compartmentalization of ClpXM2-EGFP to the mitochondria was further supported by co-localization studies using a mitochondrion-selective dye, Mitotracker® Red (Fig. 7, E–G), in which co-segregation of the EGFP fluorescence (Fig. 7E) with the rhodamine emission of Mitotracker® Red (Fig. 7F) was observed through numerous confocal sections (Fig. 7G). Similar co-localization was observed for ClpXM2-EGFP (data not shown).

**DISCUSSION**

The current study presents the cloning and initial characterization of murine ClpX, a novel member of the Hsp100/Clp family of molecular chaperones and energy-dependent protease regulators. The encoded protein of 632 amino acids is a class II member as characterized by its general structural organization as well as the presence of only one and not two nucleotide binding domains (1). Sequence homology exhibited between the E. coli, S. cerevisiae, C. elegans, and mouse members of this chaperone subfamily is concentrated toward the central and C-terminal regions of the molecules corresponding to the ATPhase and the PDZ-like substrate recognition domains. Significant amino acid sequence divergence is observed at the N terminus due to the nonconserved nature of mitochondrial targeting peptides and the obvious absence of the peptide in E. coli. An additional N-terminal difference is that only three of the homologs contain a C4-zinc finger, with its noticeable absence in S. cerevisiae (Fig. 1B). Since a ClpP homolog is also not evident in S. cerevisiae (29, 56) the C4-zinc finger of E. coli, C. elegans, and mouse ClpX is a potential candidate for mediating recruitment of ClpP. However, as is the case with DNA J, the C4-zinc binding domain may also direct recognition and binding of denatured protein substrates (47). It is interesting to speculate that ClpX may possess a bimodal capacity for substrate recognition with the N-terminal C4-zinc finger nonspecifically contacting denatured polypeptides and the C-terminal PDZ domains directing specific interactions with native proteins.

Since the molecular chaperone activity of Hsp100/Clp family members has been directly linked to their ability to hydrolyze ATP (13, 48, 49), it is noteworthy that the K, of basal ATPhase hydrolysis by ClpX is ~25 μM. This value is over 20- (low salt) to 200-fold (high salt) lower than that obtained for Hsp104 (4), 20-fold lower than that for E. coli ClpX (13), 8-fold lower than...
that for *E. coli* ClpA (48), and over 40-fold lower than the value for ClpB (50). While the basis of these differences is presently unclear, the resiliency of ClpX ATPase activity under conditions mimicking cellular stress (4) supports its membership in a Hsp100 class of stress tolerance proteins (6–9, 30, 45, 51).

The absence of multiple transcripts in all tissues but the testes and the direct correspondence of the ~2.9-kb cDNA, which we have cloned with the single ~2.9-kb transcript identified by Northern analysis, suggest the existence of a single form of ClpX in most tissues. Within this single transcript, the identification of two alternative start ATGs (M1 and M2) by *in vitro* transcription/translation experiments initially posed the exciting possibility that both mitochondrial and cytosolic forms of ClpX could be generated from the same transcript as has been documented for other proteins (52, 53). Such a mechanism for subcellular compartmentalization would be both biologically economical as well as resourceful, since it could provide a means of controlling differential compartmentalization in response to cellular metabolic status. However, GFP fusions with ClpXM1 and ClpXM2 both localized to the mitochondria and suggest that mice, like yeast, do not possess a cytosolic form of ClpX. Indeed, the truncated second form observed in the transcription/translation reactions may simply represent an aberrant product of the *in vitro* reaction.

The tissue-specific pattern of ClpX expression is inconsistent with a role as a constitutive chaperone and suggests that ClpX has acquired tissue-specific mitochondrial functions. The lack of an essential requirement of eukaryotic mitochondrial ClpX for general cell viability under normal growth conditions has been supported by the absence of an obvious phenotypic effect following disruption of the ClpX homolog in yeast (30). It is particularly interesting that mouse ClpX is most highly expressed in the liver where the mitochondria participate in numerous cell type-specific functions. These liver-specific mitochondrial processes include the oxidation of drugs and other toxic compounds, the formation of ketone bodies, the synthesis of components of fatty acid precursors, and the generation of critical components of the nitrogen metabolic pathway. Eluci-
transforms them into substrates for amino acids. Transfer of the CTS to heterologous proteins characterized by a central charged core flanked by hydrophobic acid sequence, this C-terminal targeting sequence (CTS) is final PDZ domains (3). Although not highly conserved in amino CtrA (21) through a direct interaction with the ClpX C-termi-
lar to those of C. elegans, S. cerevisiae, and E. coli, it is highly plausible that the mode and targets of substrate recognition by ClpX will also be evolutionarily conserved. To identify eukaryotic mitochondrial proteins possessing a CTS homologous to that of MuA, the Mu repressor, SsrA proteins, or CtrA, we analyzed the mitochondrial subcategory of the Yeast Protein Data base (http://www.proteome.com/YPDhome.html) (55).

Since this resource is a compilation of nearly all yeast mitochondrial genes as identified from the complete sequence of the yeast genome (29, 56), it provides a thorough representation of the entire range of potential ClpX substrates. Interestingly, of the 293 mitochondrial proteins in the data base, only nine fulfilled our criteria (see “Experimental Procedures”) for homology to the CTS within the last 11 amino acids: electron transferring flavoprotein, β chain (GenBank™ accession number 1323371), citrate transport protein 1 (GenBank™ accession number 536746), import receptors of the outer membrane TOM70 and TOM72 (GenBank™ accession numbers 1302050 and 529136), RIP1 component of the cytochrome bc complex (GenBank™ accession number 602391), NADH-ubiquinone oxidoreductase (GenBank™ accession number 805022), MSH1, the yeast homolog of E. coli MutS (GenBank™ accession number 529134), proline oxidase (GenBank™ accession number 1360564), and the E2 component of the pyruvate dehydrogenase complex (GenBank™ accession number 1301955). The first six potential substrates are in agreement with the tight inner membrane association observed for S. cerevisiae ClpX (30). This information, which suggests a possible role of ClpX in modulation of molecular import, electron transport, biochemical pathways and cell viability during normal or stressed cellular conditions, may ultimately prove valuable in elucidating its role in mammalian mitochondrial protein homeostasis.

Identification of the eukaryotic targets of mouse ClpX may be informed by parallels with the bacterial system. E. coli ClpX binds to the C-terminal 7–11 amino acids of the Mu transposase (22), of the Mu repressor (24), of the SsrA C-terminal peptide tails (18), and of the C. crescentus cell-cycle regulator CtrA (21) through a direct interaction with the ClpX C-terminal PDZ domains (3). Although not highly conserved in amino acid sequence, this C-terminal targeting sequence (CTS) is characterized by a central charged core flanked by hydrophobic amino acids. Transfer of the CTS to heterologous proteins transforms them into substrates for E. coli ClpX function (22). Since the PDZ domains of mouse are approximately 60% similar to those of C. elegans, S. cerevisiae, and E. coli, it is highly plausible that the mode and targets of substrate recognition by ClpX will also be evolutionarily conserved. To identify eukaryotic mitochondrial proteins possessing a CTS homologous to that of MuA, the Mu repressor, SsrA proteins, or CtrA, we analyzed the mitochondrial subcategory of the Yeast Protein Data base (http://www.proteome.com/YPDhome.html) (55).

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Addendum—A human homolog has been reported recently (GenBank™ accession number AJ006267 (C. Jespersgaard, P. Bross, T. J. Kjeldgaard, and Jose Trincao) and Ref. 36) for homology to the CTS within the last 11 amino acids: electron transferring flavoprotein, β chain (GenBank™ accession number 1323371), citrate transport protein 1 (GenBank™ accession number 536746), import receptors of the outer membrane TOM70 and TOM72 (GenBank™ accession numbers 1302050 and 529136), RIP1 component of the cytochrome bc complex (GenBank™ accession number 602391), NADH-ubiquinone oxidoreductase (GenBank™ accession number 805022), MSH1, the yeast homolog of E. coli MutS (GenBank™ accession number 529134), proline oxidase (GenBank™ accession number 1360564), and the E2 component of the pyruvate dehydrogenase complex (GenBank™ accession number 1301955). The first six potential substrates are in agreement with the tight inner membrane association observed for S. cerevisiae ClpX (30). This information, which suggests a possible role of ClpX in modulation of molecular import, electron transport, biochemical pathways and cell viability during normal or stressed cellular conditions, may ultimately prove valuable in elucidating its role in mammalian mitochondrial protein homeostasis.
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