A human homologue of *Escherichia coli* ClpP caseinolytic protease: recombinant expression, intracellular processing and subcellular localization

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We have recently cloned a human cDNA (hClpP) with significant sequence similarity to the ATP-dependent *Escherichia coli* ClpP protease [Bross, Andresen, Knudsen, Kruse and Gregersen (1995) FEBS Lett. 377, 249–252]. In the present study, synthesis, intracellular processing and subcellular localization of hClpP have been analysed in intact cells and in a cell-free system. Using pulse-labelling/immunoprecipitation of Chang cells transfected with the hClpP cDNA, we observed two major bands with apparent molecular masses of approx. 39 and 37 kDa. A pulse-chase experiment showed that these bands were converted into one mature-enzyme band with a molecular mass of approx. 32 kDa that was stable for at least 24 h. The 37 kDa band co-migrated with a band produced upon expression of full-length hClpP in *E. coli*, and the 32 kDa band co-migrated with the product of *E. coli*-expressed hClpP in which the 56 N-terminal residues had been deleted, indicating that the 37 kDa moiety

represents the precursor and that approx. 56 residues are cleaved off during maturation. The processing of hClpP in intact cells was dependent on mitochondrial membrane potential. These results were confirmed in an import assay system using *in vitro* transcription and translation directed by the hClpP cDNA and isolated rat liver mitochondria. No protease activity towards a series of fluorogenic peptides could be observed in extracts of Chang cells overexpressing hClpP, indicating that the protease may not be active without co-factors. Immunofluorescence studies using confocal-laser-scanning microscopy showed co-localization of the hClpP and the mitochondrially located Hsp60 (heat-shock protein 60). Taken together, the results reported here show that hClpP is localized inside mitochondria and that the trafficking and processing of hClpP resembles the typical biogenesis pathway for nuclear-encoded mitochondrial proteins.

INTRODUCTION

Controlled intracellular proteolysis is essential for cellular function, ensuring removal of damaged and misfolded polypeptides and of short-lived regulatory proteins [1]. This is a fundamental mechanism in normal cells and it is of particular relevance in many disease states. It has been recognized that impaired folding due to mutations is a frequent cause of genetic diseases [2,3]. Impaired folding leads either to accumulation of misfolded mutant polypeptides or to their rapid degradation.

Degradation of polypeptides and whole organelles by lysosomes is well known. Recent progress in the understanding of protein degradation by the proteasome in the cytoplasm [4] and its involvement in degradation of mutant proteins accumulating in the endoplasmic reticulum [5] has substantiated the notion that controlled proteolysis is of crucial importance for the functional maintenance of eukaryotic cells.

Proteases have also been detected inside mitochondria. However, much of the knowledge of proteases in eukaryotic mitochondria is based on studies of the ATP-dependent proteases from *Escherichia coli* [6,7]. Yeast and human mitochondrial homologues of the soluble *E. coli* protease Lon [8–10] and two yeast homologues of the membrane-associated *E. coli* FtsH protease [11,12] have been identified and characterized. No

homologue of *E. coli* ClpP protease has been detected in the whole yeast genome [6]. However, we have recently cloned the cDNA for a human ClpP homologue (hClpP) [13]. The hClpP cDNA (EMBL accession no Z50853) encodes a polypeptide of 277 amino acids. It displays very high similarity (53% identity) with *E. coli* ClpP in the stretch where both sequences overlap. hClpP has a long N-terminal extension with properties typical of mitochondrial targeting peptides (mTP), but the subcellular localization and intracellular processing of hClpP has not yet been investigated.

E. coli ClpP is a serine protease which forms a characteristic complex consisting of a double heptamer ring. This ring structure is similar to the architecture of the proteasome and the GroESL chaperonin complex [14,15]. The active protease consists of a double ring of ClpP subunits and either ClpA or ClpX, both of which are chaperones with ATPase activity. The co-operating subunits confer different substrate specificities and their role is apparently to present the substrate to the ClpP protease in an unfolded conformation [7]. *E. coli* ClpP alone is only capable of digesting short peptides, whereas the ClpP–ClpA complex is able to processively degrade long polypeptides into short peptides [16,17].

E. coli ClpP, together with other components, constitutes part of a protein quality control system in which chaperones promote

Abbreviations used: AAV, adeno-associated virus; AMC, 7-amido-4-methylcoumarylamide; DNP, 2,4-dinitrophenol; ClpP, caseinolytic protease; CMV, cytomegalovirus; mTP, mitochondrial targeting peptide; MPP, mitochondrial processing protease; RACE, rapid amplification of cDNA ends; Suc, N-succinylamide; Hsp60, heat-shock protein 60; MCAD, medium-chain-acyl-CoA dehydrogenase.

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folding of folding competent chains and proteases eliminate aberrant polypeptide chains [18]. hClpP may thus play a role in a mammalian protein-quality-control system. In order to provide a basis for studying this question we have investigated the subcellular localization and intracellular processing of hClpP.

EXPERIMENTAL

Plasmid constructs

For construction of a plasmid with the entire coding region of hClpP cDNA, the hClpP inserts of one of the 5'-prime clones and one of the 3'-prime clones of the cloned rapid amplification of cDNA ends (RACE) products described previously [13] were fused using the Sse8387I restriction site located in the overlap between the clones. The resulting product, pCRII-hClpP1A, comprises 31 nucleotides of 5'-untranslated sequence, the entire coding region, and the 3'-untranslated sequence, including the poly(A) tract. For expression in eukaryotic cells, the pCRII-hClpP1A fragment extending from the *Eco*RI site 5' of the insert to the *Dra*I site at position +1002 of the hClpP cDNA sequence was purified, filled in with Klenow and ligated into the expression plasmid pMP6 [an AAV (adeno-associated virus) vector containing a CMV (cytomegalovirus) promoter kindly provided by Dr. Ramila Philip, Applied Immune Sciences Inc., Santa Clara, CA, U.S.A.] and a product with the sense orientation relative to the CMV promoter [pMP6-hClpP] was chosen. For *in vitro* translation, the pCRII-hClpP1A plasmid was digested with *Eco*RI and the large fragment was re-ligated with the fragment comprising the hClpP cDNA insert. This treatment deletes part of the sequence added during the RACE procedure and a plasmid with the orientation allowing transcription of hClpP from the SP6 promoter was selected (pCRII-hClpP-SP6). Plasmids for expression of full-length and an N-terminally truncated hClpP variant (hClpP56) in *E. coli* were constructed by PCR using primers introducing a *Nde*I site comprising an ATG codon immediately 5' to the codons for amino acids 2 and 56 respectively. The PCR products were cloned as *Nde*I/*Bam*HI fragments into the pBluescriptKS⁻ (Stratagene)-based medium-chain-acyl-CoA dehydrogenase (MCAD) expression plasmid pWT [19] replacing the MCAD insert with the hClpP insert. For antibody production a fusion protein of glutathione S-transferase with hClpP (amino acids 48–277) was constructed. For this purpose PCR was performed with pCRII-hClpP1A as template and a primer introducing a *Bam*HI site at position +137 and a primer in the polylinker adjacent to the 3'-end of hClpP. The PCR product was cleaved with *Bam*HI and *Dra*I and the resulting fragment introduced into the glutathione S-transferase fusion vector pGEX5X (Pharmacia). The fusion protein was expressed and purified on a glutathione S-transferase column (Pharmacia) as recommended by the supplier and used to raise a polyclonal antibody in rabbits. The antiserum was purified on a Protein G column, and an aliquot was further purified by passing it through a column coupled with a total *E. coli* protein extract prepared from *E. coli* expressing the pGEX5X vector without insert. *E. coli* ClpP was amplified by PCR from genomic DNA of strain MC4100 [20] using a primer that introduces a *Nco*I site overlapping with the ATG start codon and a 27-mer antisense primer complementary to nucleotides 1006–980 of the published sequence [21]. The PCR fragment was cloned into the vector pCR-Script SK⁺ (Stratagene). The product was checked for the absence of PCR errors by sequencing and the *E. coli* ClpP insert was subcloned as *Nco*I/*Xho*I fragment into a derivative of pTrc99B [22] into which the coding sequence for mature human MCAD had been introduced. The final product contains *E. coli* ClpP

under control of the hybrid *trp/lac*-promoter forming an operon with the MCAD gene which follows further downstream.

Expression of hClpP in eukaryotic and prokaryotic cells

Eukaryotic Chang and COS-7 cells [both from the American Type Culture Collection (A.T.C.C.), Rockville, MD, U.S.A.] were either grown in 75 cm² flasks, slideflasks or 10 cm² dishes (Nunc, Roskilde, Denmark) at 37 °C and 5% (v/v) CO₂ in RPMI 1640 (In Vitro, Copenhagen, Denmark) containing 10% (v/v) FCS [GIBCO-BRL]. DNA transfection of eukaryotic cells with pMP6-hClpP was accomplished using the calcium phosphate co-precipitation method [23,24]. As a negative control for hClpP expression we used the vector without the hClpP cDNA insert. All cells were examined between 36 and 48 h post transfection. In some experiments, cells were fixed in freshly prepared 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde (both from Merck) for 5 min at 4 °C, followed by one wash in PBS. The cells were then permeabilized with 70% (v/v) ethanol for 30 min at –20 °C. Expression in prokaryotic cells was performed in *E. coli* JM109 or SG12050 [25,26] transformed with the respective expression plasmid. Cells were grown at 31 °C in 2 × TY medium [27] supplemented with 100 µg/ml ampicillin to a *D*₅₃₂ of 0.4–0.8 and induced with 1 mM isopropyl thio-galactoside for 3 h. Cells were harvested and disrupted as described in [28] with the exception that Tris/HCl, pH 8.0, was replaced with triethanolamine, pH 8.0, in the solutions used for lysis of the bacterial cells.

Gel electrophoresis

SDS/PAGE was carried out with ReadyGels (Bio-Rad) according to the recommendations from the supplier. Western blotting was performed as described previously [28]. Native PAGE was performed with 4–15% ReadyGels (Bio-Rad), omitting SDS in the running buffer.

Measurement of proteolytic activity towards fluorogenic peptides

Fluorogenic peptides were obtained from Sigma. Extracts were prepared from Chang cells by sonication of cell pellets for 20 s (micro tip; 50% duty cycle; maximum setting) in buffer L [103 mM triethanolamine/66 mM EDTA/5.9% (w/v) sucrose/0.117 mg/ml lysozyme/0.06% (v/v) Triton X-100, pH 8.0], followed by centrifugation for 5 min at 4 °C and 10000 *g*. The supernatant containing soluble proteins was withdrawn and stored at –20 °C until used. Proteolytic activity towards fluorogenic peptides was determined as described by Woo et al. [29] with minor modifications: 60 µl of reaction mixture, comprising total soluble protein extract, buffer and the respective fluorogenic peptide, was prepared on ice and 25 µl was withdrawn and quenched with SDS/sodium borate. The remainder of the reaction mixture was incubated at 37 °C and 25 µl was withdrawn and quenched in the same way after 30 min incubation time. Control experiments indicated that the measured activity was linear with time for at least 50 min and linear with the protein concentration over the ranges used. Fluorescence in the samples withdrawn before and after incubation at 37 °C was measured with a Perkin–Elmer LS50B luminescence spectrometer (excitation 380 nm, emission 440 nm). The difference between the values obtained before and after incubation was calculated/min per mg of total soluble protein.

***In vitro* transcription and translation**

The pCRII-hClpP-SP6 construct was used in the TNT[®]-coupled transcription/translation rabbit reticulocyte-lysate system (Promega) for coupled *in vitro* transcription and translation with [³⁵S]methionine (Amersham International) according to the protocol provided by the manufacturer. Transcription was carried out using SP6 RNA polymerase, and translation was allowed to continue for 120 min at 30 °C. Protein synthesis was arrested by adding 150 µg/ml cycloheximide (Sigma) [30], and the translation products were used immediately for the import assay.

Mitochondrial import *in vitro*

Mitochondria from female adult WISTAR rats were prepared as described by Kølvrå and Gregersen [31]. The import assay was initiated by mixing 10 µl of the *in vitro* translation reaction mixture and 6 µl of freshly made mitochondria (8 mg total protein/ml), and the samples were incubated for 45 min at 27 °C with constant shaking [30]. After incubation the samples were centrifuged at 12000 *g* for 5 min and supernatants and pellets collected. Pellets were washed twice in ice-cold buffer A containing 225 mM mannitol, 25 mM sucrose, 10 mM Tris/HCl, pH 7.4, and 0.1 mM EDTA [31]. In some experiments mitochondria were preincubated with 200 µM of the uncoupler 2,4-dinitrophenol (DNP) before addition to the translation mixture. In the trypsin-protection assay, 0.5 µg/µl trypsin (Gibco-BRL) was added to the samples after the import reaction was completed, followed by 10 min incubation at 4 °C.

Mitochondrial import *in vivo*

Chang cells in 10 cm² dishes were transfected with the hClpP construct and cultured at 37 °C. At 24 h post transfection the cells were labelled with [³⁵S]methionine and [³⁵S]cysteine (50 µCi; Amersham) in 500 µl of labelling medium according to standard procedures [32]. The dishes were incubated at 37 °C for 15 min. After removing the labelling medium, cells were rapidly washed twice with PBS and then harvested with a plastic scraper in 1 ml of medium containing no label. Cells were washed twice in ice-cold PBS, and then stored at –70 °C. When Rhodamine 6G was used, it was added to the medium at a final concentration of 2.1 µM, 30 min before the medium was replaced with the medium containing label [33,34]. The cells were then incubated at 37 °C for 15 min in the labelling medium containing the same concentration of Rhodamine 6G. Cells in the chase experiment were labelled as described above. After a 15 min pulse, the labelling medium was removed and cells were rinsed twice in PBS. Then 1 ml of medium containing no label was added to each dish, and the cells were harvested as described above at 0.5, 1, 2, 4, 8 and 24 h.

Immunoprecipitation of radiolabelled hClpP proteins from Chang cells

The frozen pellets were resuspended in 100 µl of ice-cold lysis buffer [35], placed on ice for 1 h and centrifuged for 30 min at 4 °C and 10000 *g*. The supernatant was cleared by adding 10 µg of irrelevant rabbit antibody (Sigma) and incubated for 1.5 h at 4 °C, followed by addition of 25 µl of a 1:1 slurry of Protein A-Sepharose dilution (Pharmacia) and further incubation at 4 °C for 1.5 h. The Protein A-Sepharose complexes were collected by centrifugation for 3 min at 4 °C and 200 *g*. The cleared supernatant was then incubated with 10 µg of anti-hClpP antibody for 12 h at 4 °C and hClpP-antigen-antibody complexes

were recovered by adding Protein A-Sepharose followed by centrifugation. The pellets were washed twice in 200 µl dilution buffer I [50 mM Hepes (pH 7.4)/100 mM NaCl/0.1 % (v/v) Triton X-100/10 mM EDTA/10 mM EGTA/2.2 % (v/v) DMSO/10 mM *N*-ethylmaleimide/0.1 % (w/v) haemoglobin] and then twice in 200 µl of dilution buffer II (same as dilution buffer I, but without Triton X-100). The immunoprecipitated complexes were solubilized in 20 µl of dilution buffer II and boiled for 5 min with an equal volume of buffer containing 8 % (w/v) SDS, 1 % (w/v) Bromophenol Blue, 17 % (v/v) glycerol, 0.125 M Tris/HCl, pH 6.8, and 0.1 M dithiothreitol. The samples were subjected to SDS/PAGE and the gels were treated for fluorography with Amplify (Amersham) according to the protocol provided by the manufacturer.

Immunostaining and localization of hClpP and Hsp60 by confocal-laser-scanning microscopy

A two-layer immunostaining procedure was used with three washing steps in PBS between each incubation. Fixed cells were incubated for 60 min at room temperature with rabbit anti-hClpP primary antibodies. Then the cells were incubated for 60 min with fluorescein-conjugated porcine anti-rabbit secondary antibodies (Dako, Copenhagen, Denmark). Cells stained in order to detect both hClpP and mitochondria were also incubated with mouse anti-(human Hsp60) primary antibodies (Sigma) followed by Rhodamine-conjugated goat anti-mouse secondary antibodies (Sigma). Controls for the Hsp60 staining were incubated in equal concentrations of serum from a non-immunized mouse, followed by Rhodamine-conjugated goat anti-mouse secondary antibodies.

RESULTS

Recombinant expression of human ClpP

Sequence alignment of hClpP with the amino acid sequence of *E. coli* ClpP showed that hClpP possesses an N-terminal extension of 42 amino acids [13]. This extension displays properties of typical mTPs [36,37], e.g. enrichment of arginine residues and absence of negatively charged residues (Figure 1). Stretches within this extension have the potential to form an amphiphilic α -helix (results not shown). This suggests that hClpP contains an mTP. Using the rules described by Gavel and von Heijne [37], the putative cleavage site for mitochondrial processing protease (MPP) was localized. Since neither an R-X-Y-(S/A) nor an R-X-F-S motif could be found, the putative cleavage site was assumed to be localized between the first negatively charged residue (Glu⁶⁴) and the closest upstream arginine residue (Arg⁵⁴). As this arginine residue is typically located at position –2, the cleavage site was predicted to be between Ala⁵⁵ and Leu⁵⁶. In the alignment this is close to the start point for mature *E. coli* ClpP after autocatalytic cleavage of its 14 N-terminal amino acids. The predicted N-terminus of mature hClpP is localized at the beginning of the region displaying high sequence similarity to other ClpP homologues (Pro⁶⁰ is the first residue conserved in a series of ClpPs from different organisms) [13].

To substantiate the proposed localization of the cleavage site, plasmids comprising the entire hClpP coding region and an N-terminally truncated variant of hClpP (Figure 1) were constructed. hClpP cDNA was expressed in eukaryotic Chang and COS-7 cells (see the Experimental section) and the precursor and the N-terminally truncated variant were expressed in *E. coli*. Total soluble protein extracts from eukaryotic and prokaryotic

Table 1 Analysis of proteolytic activities towards fluorogenic peptides

Extracts from Chang cells transfected with the control vector or a plasmid containing hClpP cDNA as indicated were analysed for protease activity as described in the Experimental section. As a control, extracts from *E. coli* cells transformed with a plasmid encoding *E. coli* ClpP or the vector were analysed in the same way. Values give the change in fluorescence (arbitrary units)/min per mg of soluble protein extract. The values represent two measurements.

Peptide substrate	Chang		<i>E. coli</i> SG12050 (<i>clpP</i> ⁻)	
	Vector	hClpP	Vector	<i>E. c.</i> ClpP
Suc-Leu-Tyr-AMC	440/535	407/50	< 100	27436/32234
Suc-Ala-Ala-Ala-AMC	98/142	80/91	< 100	1309/889
Suc-Leu-Leu-Val-Tyr-AMC	1043/1769	951/1425	< 100	674/413
Suc-Ala-Ala-Phe-AMC	1332/1245	1176/1202	< 100	873/651
Suc-Ile-Ile-Trp-AMC	171/142	123/223	< 100	2031/1571
Suc-Gly-Pro-AMC	551/530	334/483	< 100	< 100
Suc-Ala-Phe-Lys-AMC	164/268	247/157	3449/4846	1261/1277

also displays such an activity, extracts from Chang cells transfected with the hClpP cDNA construct and controls were analysed for cleavage of a series of fluorogenic peptides (Table 1). Chang cells display different levels of endogenous activity towards all the substrates tested. The values for the various substrates are comparable because all substrates contain AMC as the fluorescent group that is released by proteolytic cleavage. No elevated proteolytic activity towards any of the peptides tested in comparison with the control could be observed (Table 1) in the extracts from the cells transfected with hClpP cDNA. Western blotting of aliquots from the extracts revealed the presence of hClpP protein (Figure 2A, lane 2; Figure 2B, lane 2). Overexpression of *E. coli* ClpP in *E. coli clpP*⁻ produced highly elevated activity levels towards the peptide Suc-Leu-Tyr-AMC and moderately elevated activity levels towards the other substrates, except for Suc-Ala-Phe-Lys-AMC. The fact that the *E. coli* extracts were prepared in the same buffer indicates that the assay system can reveal protease activity. Still, no elevated activities compared with controls towards any of the fluorogenic peptides were detected in extracts from COS-7 cells overexpressing hClpP cDNA and in *E. coli* cells expressing full-length hClpP or the N-terminally truncated variant (results not shown). Taken together, these data may indicate that hClpP alone (unprocessed or processed) does not cleave any of the substrates tested.

Import analysis of hClpP into rat liver mitochondria *in vitro* and in intact Chang cells

In order to investigate the processing of hClpP we used an *in vitro* mitochondrial import system. Transcription and translation of hClpP cDNA resulted in the synthesis of a major protein of approx. 37 kDa. (Figure 3A, lane 1). This product was consistent with the size of the precursor band detected in transfected COS-7 or Chang cells at steady state (Figure 2A, lanes 2 and 9). The product was incubated with purified rat liver mitochondria at 27 °C for 45 min, followed by centrifugation. In the supernatant solution the amount of precursor hClpP was decreased as compared with a sample incubated without mitochondria (Figure 3A, lanes 1 and 2). In the mitochondrial pellet we observed a product representing the mature hClpP form (Figure 3A, lane 3) with an apparent molecular mass identical with that of the mature band (m) of hClpP from transfected Chang or COS-7 cells (Figure 2A, lanes 2 and 9). We found that the processing

Figure 3 Import of hClpP into mitochondria *in vitro* and in intact Chang cells

(A) Mitochondrial import *in vitro*. Coupled *in vitro* transcription/translation with [³⁵S]methionine was performed as described in the Experimental section. The product from the *in vitro* translation was incubated with purified rat liver mitochondria for 45 min at 27 °C in the presence or absence of 200 μ M DNP as indicated. Mitochondria were collected by centrifugation following two washing steps in buffer A. To monitor the processing of hClpP, equivalent aliquots of the translation products in the supernatant (sup) and the pelleted mitochondrial fraction (pellet) were subjected to SDS/PAGE and fluorography. Uptake into mitochondria was tested by treating supernatant and pellet with trypsin after the import reaction was completed. The following lanes were loaded: lane 1, product of the *in vitro* transcription/translation; lane 2, supernatant fraction after incubation of the translation product with rat liver mitochondria; lane 3, pellet fraction after incubation of the translation product with rat liver mitochondria; lane 4, supernatant fraction after incubation of the translation product with rat liver mitochondria in the presence of DNP; lane 5, pellet fraction after incubation of the translation product with rat liver mitochondria in the presence of DNP; lane 6, supernatant fraction after incubation of the translation product with rat liver mitochondria followed by trypsin treatment; lane 7, pellet fraction after incubation of the translation product with rat liver mitochondria followed by trypsin treatment. (B) Mitochondrial import of hClpP in intact Chang cells. The metabolic labelling of transfected Chang cells with [³⁵S]methionine and [³⁵S]cysteine followed by immunoprecipitation is described in the Experimental section. Lane 1, hClpP from transfected Chang cells metabolically labelled in the presence of Rhodamine 6G; lane 2, hClpP from transfected Chang cells metabolically labelled in the absence of Rhodamine 6G. The positions and molecular masses in kDa of co-electrophoresed marker proteins are indicated on the left, and positions of the additional band (p*), precursor (p) and mature (m) hClpP are shown on the right. These results are representative of three separate experiments.

was not complete, since hClpP precursor was also found with the washed mitochondrial pellet (Figure 3A, lane 3).

In order to investigate whether the processing of the hClpP precursor to the mature form in the presence of mitochondria was dependent on a potential across the mitochondrial membrane, the import assay was carried out in the presence or absence of DNP, an uncoupler of the proton electrochemical gradient that inhibits mitochondrial uptake of precursor polypeptides. When adding 200 μ M DNP to the import assay, the processing of the

Figure 4 Pulse-chase analysis of hClpP import into mitochondria in Chang cells

Transfected Chang cells were metabolically labelled for 15 min and chases from 0.5 to 24 h (as indicated) were performed as described in the Experimental section. Positions and molecular masses in kDa of co-electrophoresed marker proteins are indicated on the left. The positions of the additional band (p*), precursor (p) and mature (m) hClpP are indicated on the right. These results are representative of three separate experiments.

precursor was completely blocked (Figure 3A, lanes 4 and 5). To test the mitochondrial uptake of hClpP precursor, trypsin was added to the samples after the import reaction. Only the mature form of hClpP was protected from trypsin digestion, whereas the precursor was almost totally digested (Figure 3A, lanes 6 and 7). Owing to the fact that the precursor hClpP molecules observed in the pellet fraction were removed by trypsin treatment (Figure 3A, lanes 3 and 7), we suggest that these hClpP molecules were positioned on the outside of the mitochondrial outer membrane, probably associated with the import machinery.

To determine whether the processing of hClpP observed *in vitro* in the mitochondrial import assay reflects the processing in intact cells, pulse-labelling experiments using [35 S]methionine and [35 S]cysteine metabolic labelling in transfected Chang cells followed by immunoprecipitation were performed. Cells were labelled for 15 min in the presence or absence of Rhodamine 6G, an inhibitor of the mitochondrial uptake of precursor polypeptides [40]. In the presence of 2.1 μ M Rhodamine 6G, two products (p and p*; Figure 3B, lane 1) were observed with

approximately the same molecular mass as the p and p* bands observed by Western blotting (Figure 2, lanes 2 and 9). The product with the lowest molecular mass co-migrates with the precursor (p) synthesized *in vitro* (results not shown). The band representing the mature form of hClpP was not detectable, showing that the *in vivo* processing of hClpP was membrane-potential-dependent, in accordance with the data observed *in vitro*. When pulse-labelled in the absence of Rhodamine 6G, a band representing the mature form was observed (Figure 3B, lane 2). This band co-migrates with the processed product observed *in vitro* (results not shown). None of the hClpP bands were detected when cells were transfected with a vector without hClpP cDNA insert (results not shown), illustrating that the observed bands indeed represent products of hClpP.

To study the kinetics of processing of the hClpP precursor in transfected Chang cells we performed pulse-chase/immunoprecipitation experiments. Cells were labelled for 15 min, and chases from 0.5 to 24 h were performed (Figure 4). The amount of radiolabelled precursor (p) and the high-molecular-mass product (p*) clearly decreased with time, had almost disappeared after 4 h and could not be detected after 8 h. Concomitantly the intensity of the band corresponding to the mature form of hClpP increased. The ratio between the p and p* bands remained fairly constant throughout the experiment. The mature form was stable for at least 24 h.

Mitochondrial localization of hClpP

The fate of hClpP was analysed in intact Chang cells by *in situ* immunostaining. The anti-hClpP-antibody labelling resulted in a predominant staining of typical mitochondrial structures, very similar to cells stained by the mitochondria specific dye Rhodamine 6G. To study the specific intracellular localization of hClpP at high resolution, we performed double labelling with the anti-hClpP antibody and a monoclonal antibody directed against human Hsp60 followed by confocal-laser-scanning-microscopical analysis (Figure 5). Our results showed that the hClpP immunoreactivity co-localizes with the immunoreactivity pro-

Figure 5 Co-localization of hClpP and Hsp60 protein in transfected Chang cells revealed by confocal-laser-scanning microscopy

(A) shows the hClpP labelling in a focal section of Chang cells transfected with a plasmid harbouring the hClpP cDNA sequence where fluorescein (green)-labelled antibodies indirectly immunostain hClpP. Nuclear DNA is counterstained with Hoechst 33258 (blue). The same image for endogenously expressed Hsp60 is shown in (B), where Rhodamine (red) fluorescence represents antibodies revealing Hsp60 in mitochondria. (Σ) illustrates that the hClpP and Hsp60 immunoreactivity co-localize inside mitochondria. Analysis of other focal sections of the same image further established that the hClpP immunoreactivity co-localizes with immunoreactivity produced by anti-hsp60 antibodies (results not shown). The results are representative of three separate experiments.

duced by anti-Hsp60 antibodies. Inspection of different confocal sections (results not shown) showed complete co-localization between hClpP and Hsp60 immunoreactivity demonstrating that hClpP and Hsp60 co-localize in mitochondria.

DISCUSSION

The present results show that the hClpP protein is synthesized in the cytosol as a precursor molecule, imported into mitochondria in a membrane-potential-dependent reaction and processed by intramitochondrial proteases to the mature form. This illustrates that the trafficking and processing of hClpP resembles the typical biogenesis pathway for nuclear-encoded mitochondrial proteins. Evidence for this model is based on the facts (1) that in the presence of purified rat liver mitochondria the hClpP precursor produced *in vitro* is processed to a form which is indistinguishable from that of mature hClpP identified *in vivo* in intact Chang cells, as demonstrated by pulse-labelling/immunoprecipitation experiments and Western-blotting assays, (2) that the processing of hClpP precursor is membrane-potential-dependent, since import was blocked when Rhodamine 6G or DNP were added, as shown by pulse-labelling/immunoprecipitation in intact cells and by the *in vitro* import assay, (3) that the processed mature hClpP is located inside mitochondria, presumably inside the matrix compartment, illustrated by the trypsin-protection assay and the *in situ* co-localization, and (4) that there is a time-dependent decrease of hClpP precursor and a concomitant increase in the mature hClpP, as demonstrated by pulse-chase/immunoprecipitation experiments.

In intact cells, but not in the *in vitro* import assay, a band (p*) migrating slightly more slowly than the precursor (p) protein was observed. The ratio between this band and the precursor band was unchanged throughout the pulse-chase experiment, indicating that p* is not an obligatory intermediate in the processing event. The fact that it migrates more slowly than the 'real' precursor suggests that it represents a covalently modified form of the precursor. The nature of this modification remains to be determined. It is furthermore unclear whether this band is an artefact due to overexpression of hClpP or whether it represents a form which is present also under normal conditions.

Another unexplained finding was that COS-7 cells processed the hClpP precursor very inefficiently, leading to retention of high amounts of the unprocessed precursor at steady state. *In situ* immunostaining showed that the hClpP material in the COS-7 cells co-localized with mitochondrial proteins (results not shown), suggesting that hClpP is present inside the mitochondria or associated with the mitochondrial import system. We have previously used COS-7 cells for expression of other mitochondrial matrix proteins [24,41], and these proteins are processed correctly in spite of very high expression levels. It is therefore improbable that the capacity of MPP in COS-7 cells is rate-limiting. Processing of the hClpP precursor might depend on a mitochondrial cofactor or a protease other than MPP, possibly hClpP itself. It is known that *E. coli* ClpP autocatalytically cleaves off the 14 N-terminal residues [42]. This processing also occurs in ClpP-deficient *E. coli* cells in which no pre-existing ClpP is present. The fact that full-length hClpP expressed in *E. coli* is unprocessed strongly suggests that processing of hClpP is not autocatalytic. The finding that the hClpP precursor remains associated with mitochondria in the *in vitro* import assay, even after the washing procedure, illustrates that these molecules are bound to the outer surface of the organelle, probably to receptors of the import machinery. This further supports the proposed model in which the mature hClpP is processed inside the mitochondria rather than on the mitochondrial outer membrane.

We therefore suggest that processing of the hClpP precursor is not autocatalytic but dependent on MPP, possibly in co-operation with other intramitochondrial cofactors which are limited in COS-7 cells but present in appropriate amounts in Chang cells.

We were unable to detect any significantly elevated activity towards fluorogenic peptides in extracts from cells overexpressing hClpP (Table 1). One possible explanation for the lack of activity towards these substrates is that hClpP displays a sequence specificity that is not covered by the peptides used. An alignment of the amino acid sequences of human and *E. coli* ClpP shows that the amino acid residues forming the active-site triad (Ser-His-Asp) are conserved [13], suggesting that hClpP is a serine protease. A more plausible explanation for the failure to detect any elevated protease activity would be that hClpP is obligatorily dependent on a cofactor (ClpA/ClpX). Association with one of these co-factors is also necessary for degradation of larger proteins by *E. coli* ClpP protease [16]. ClpA or ClpX have been proposed to provide a sort of 'corkscrewing' effect, thus moving the protein substrate into the axial opening of the ClpP complex in order to bring it into contact with the proteolytic active site [14]. It has been observed that *E. coli* ClpP has an architecture with a central axial pore [43]. The active site is expected to reside inside the inner cavity of the ClpP cylinder and in this way sequestered from the bulk solution. It may be imagined that the axial pore of hClpP, in contrast with that of *E. coli* ClpP, is closed and has to be actively opened by a cofactor, e.g. ClpX/A. As mentioned above, the proteasome complex has a double-ring architecture similar to that of ClpP, with sequestration of the active site in the inner cavity. Comparison of the X-ray structures of the yeast and archaeobacterial proteasomes revealed that the archaeobacterial homologue has an axial opening, whereas the yeast proteasome does not [44,45], suggesting that the eukaryotic proteasomes have a stricter requirement to make the active site accessible.

Recently evidence has accumulated that the pathogenic effect of a number of human disease-causing mutations is to compromise folding [2,3]. In mitochondria, as well as in other compartments of the cells where protein folding occurs, this may lead to either accumulation or rapid degradation of malformed polypeptide chains. We have observed that many of the mutations detected in deficiencies of mitochondrial acyl-CoA dehydrogenases cause impaired folding of the mutant proteins resulting in premature degradation of folding intermediates [13,28,41,46]. We have evidence that the extent of degradation of mutant mitochondrial matrix polypeptides can be modulated (T. J. Corydon, unpublished work), resulting in variable amounts of residual functional mutant enzymes. Variations in the intra-mitochondrial balance between chaperones and proteases, constituting a protein-quality-control system, may be the explanation for this finding. The present results provide a basis for investigating whether hClpP is part of such a mitochondrial protein-quality-control system in human cells.

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