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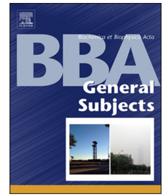

CLPB (caseinolytic peptidase B homolog), the first mitochondrial protein refoldase associated with human disease

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In 2015 we identified biallelic variants in *CLPB* gene underlying a multi-system disease presenting predominantly neurological phenotype ranging from nonprogressive intellectual disability to prenatal encephalopathy with progressive brain atrophy, movement disorder, cataracts and also neutropenia that can develop into leukemia [1]. Currently more than 35 affected individuals with *CLPB*-defect (MEGCANN syndrome, MIM#616271) are known [2–5].

The human CLPB protein, encoded by the *CLPB* gene, is named after its bacterial analog, the ClpB (*caseinolytic peptidase B*) chaperone from the HSP100 family, with which it shares significant sequence identity in the C-terminal part. This name is however misleading, as ClpB protein does not possess proteolytic activity. The microbial Hsp100 chaperones, belonging to the AAA+ ATPase family, form hexameric ring-shapes assemblies. In co-operation with Hsp70 family protein and its co-chaperones, they utilize ATP hydrolysis energy to disentangle polypeptide chains from amorphous aggregates by threading them along the hexamer's central pore, allowing for their efficient refolding [6]. However, the N-terminal part of human CLPB and microbial disaggregases share no significant similarity. Also, the first nucleotide binding domain (NBD1): an ATPase domain with its regulatory M subdomain which is crucial for disaggregase function, is missing in human CLPB. Instead, in the N-terminal part of human CLPB ankyrin repeat motifs can be distinguished (Supp. Fig. 1A). In humans CLPB is localized in the mitochondria [1]. CLPB-deficient individuals show 3-methylglutaconic aciduria, a strong biomarker for mitochondrial dysfunction, however oxidative phosphorylation activities are not affected [1,3]. While the exact role of CLPB in the cell remains elusive, there are references suggesting its involvement in regulating apoptosis, cristae maintenance [7] and mitochondrial-mediated antiviral innate immunity [8].

Here, we present for the first time the biochemical characteristics of the human CLPB protein with the aim of shedding light on its physiological function and the pathogenesis of MEGCANN syndrome.

CLPB protein (93–707 fragment, corresponding to the protein without mitochondrial signal peptide) was purified from *E. coli* expression system as the cleavable fusion with maltose binding protein (MBP) (Supp. Fig. 1B, detailed methodology in supplementary material). The circular dichroism (CD) spectrum characteristic for mostly helical proteins (Supp. Fig. 1C), and the clear melting curve both in the

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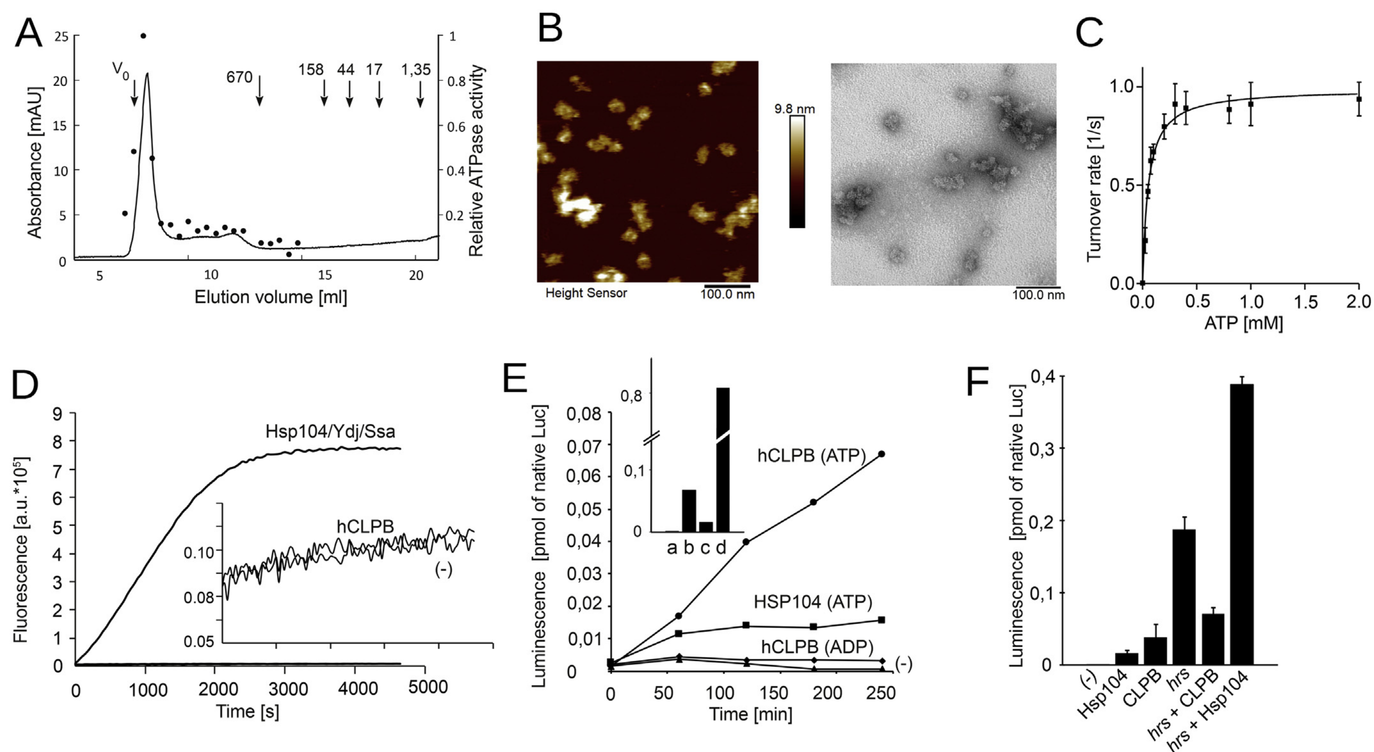

Fig. 1. A. Molecular gel filtration on Superose 6 Increase 10/300 column. Together with chromatogram UV trace, relative ATPase activity in fractions is shown (circles). ATPase activity was assayed in 0.4 mL fractions and normalized to the highest reading. Indicated by arrows are the positions of V_0 of the column (plasmid DNA) and molar masses of BioRad gel filtration standards.
B. Molecular imaging of CLPB sample. Left, atomic force microscopy, right, electron microscope, negative staining.
C. ATPase turnover rate Michaelis-Menten plot. Rates were determined in an enzyme-coupled assay (see supplement for details).
D. Reactivation of thermally denatured GFP. Inlet: magnification of fluorescence traces of denatured GFP treated with buffer (−) or human CLPB/ATP
E. Reactivation of chemically-unfolded luciferase. Reactions were performed as described in supplementary information, in the presence of ATP with creatine kinase/phosphocreatine ATP regeneration system, or ADP as indicated. Plot, the time course of reaction, inlet – the luciferase activity at the final time point (4 h): a) luciferase alone, or incubated with b) human CLPB/ATP, c) Hsp104/ATP, d) Hsp104/Ssa/Ydj/ATP. Note broken axis.
F. Renaturation of chemically unfolded luciferase. 5 h timepoint of renaturation reaction (no ATP regeneration system). Aggregated luciferase was incubated alone (−) or, as indicated, with addition of human CLPB, Hsp104, human refolding system consisting of JA2 and Hsc70 proteins (*hrs*).

CD signal and in tryptophan fluorescence, with a deflection point at 60 °C, (Suppl. Fig. 2) confirm the native conformation of the protein preparation. Molecular gel filtration experiments showed a uniform CLPB peak at a very high molecular mass (in MDa range), soon after the void volume of the Sepharose 6 increase 100/300 column, with co-localized ATPase activity (Fig. 1 A). Atomic force microscopy (AFM) and negative staining EM imaging of the protein sample showed particles of approx. 30 nm diameter (Fig. 1B). This indicates that human CLPB forms assemblies larger than hexamers similar to Hsp100 disaggregases. Although further structural studies are needed to verify the identity of such assemblies, one may speculate that at least double hexamers are formed and that presence of empty space inside might lead to the large apparent diameter of the whole assembly.

The ATPase activity of the human CLPB was already shown in our pioneering work [1]. Here, the soluble CLPB protein obtained using a different approach not only allows its independent confirmation, but also for more detailed analysis including establishing the K_m and V_{max} parameters (Fig. 1C). The ATPase turnover rate, although relatively moderate (V_{max} = 0,99 ± 0,03 1/s), is characterized by a high affinity, K_m = 0,053 ± 0,006 mM.

To address the physiological functions of CLPB, its putative disaggregase activity was examined in two different assays. In the thermally denatured GFP assay, easily renatured by the concerted action of microbial Hsp100/Hsp70 disaggregase system, human CLPB exhibited no detectable activity. (Fig. 1D) However, in the second assay we observed ATP-dependent refolding activity towards the urea-unfolded luciferase, aggregated by rapid dilution of urea (Fig. 1E). This activity, albeit slow, is clearly detectable: 50 pmol of human CLPB was able to refold 0,067 pmol of denatured luciferase (1.34% of the total luciferase amount or 7.4% of Hsp104/Ssa/Ydj-recoverable luciferase) over the course of 4 h. Comparably, the yeast disaggregase system: Hsp104 disaggregase with its partners Ssa/Ydj was able to recover the maximal amount of 0.91 pmol of luciferase within the first hour (18.1% of total luciferase present in the reaction). Human CLPB was not able to cooperate with Ssa/Ydj yeast co-chaperones (data not shown). We have also examined CLPB cooperation with a known human disaggregation system comprising of Hsc70 and JA2 proteins (members of Hsp70 and Hsp40 families, respectively) [9]. This human renaturation system was able to refold luciferase much more efficiently than CLPB alone (Fig. 1F). Addition of yeast Hsp104 disaggregase to Hsc70/JA2 led to over twofold increase of renaturation efficiency, indicating strong synergistic effect. When Hsc70/JA2 system was supplied with human CLPB protein, 2.7-fold decrease of luciferase renaturation was found relative to Hsc70/JA2 alone.

Due to the high identity between C-terminal AAA+ nucleotide binding domains of human CLPB and microbial disaggregases it is tempting to assume that the physiological function is also similar. However, no direct evidence for disaggregation/reactivation activity for human CLPB was ever presented. Evolutionary analysis reveals that the Hsp100 disaggregase family, comprising both cytosolic and mitochondrial proteins, was lost as early as the origin of metazoa [10]. The human disaggregase system, (also working on a long time scale, similar to the data presented on Fig. 1D) involve Hsp110, Hsp70 and Hsp40 chaperones, but no AAA+ protein [11].

The relatively moderate refolding efficiency compared to the microbial system may be due to a missing unknown interaction partner, or sub-optimal reaction conditions. Another explanation may be that human CLPB has a preference for specific protein(s). Human Hsc70/JA2 chaperones have a relatively low species specificity thus allowing them to enable yeast Hsp104 disaggregation activity. In combination with CLPB, however, they fail to do so (Fig. 1F). The decrease of disaggregation activity upon CLPB addition may result from competition between Hsc70/JA2 and the less-productive CLPB at aggregate binding stage. Alternatively, CLPB may persistently bind the luciferase refolding intermediates released by Hsc70/JA2. The difference in refolding capacity of luciferase and GFP substrates suggests that CLPB physiological role is not aimed at general unfolded protein maintenance. This would contrast with microbial disaggregases, whose function is mainly conferring thermotolerance to the cell via reactivation of various aggregated proteins in a non-specific manner. It is not clear, whether the CLPB preference for luciferase is due to recognition of a specific sequence or to the difference in the physical state of the two substrates (amount of secondary structure remaining, aggregate size etc.). This would suggest that CLPB may have specific preferred substrates in the cell.

The AAA+ group that contains both Hsp100 disaggregases and human CLPB protein, has many representatives with various diverse activities [12]. The common structural motif, presence of one or two typical conserved ATPase modules, confers a specific mode of action: AAA+ proteins usually form hexameric ring-shaped assemblies with a central channel, through which a substrate is being threaded in an ATP-dependent way. This may, however, be applied in a broad spectrum of processes, involving, apart from renaturation of aggegates, also extracting polypeptides for downstream proteolysis (ClpA, ClpX protease regulatory subunits), proteolysis itself (FtsH or Lon proteases) or even DNA remodeling (RuvB recombinase subunit). These diverse functions depend on the kind of additional domains present in conjunction with the AAA+ ATPase motor module. Although the human CLPB D2 subdomain, at its C-terminal end is typical for disaggregases, the ankyrin region that precede AAA+ ATPase in human CLPB is unprecedented. Nothing so far can be inferred from its presence with regard to the protein's function.

Recently human CLPB was reported to be interacting with the prohibitins complex (PHB) in the mitochondrial intermembrane space [8]. It may well be that CLPB fulfills specific substrate threading in that complex. The prohibitins PHB1 and PHB2 are inner mitochondrial membrane proteins forming a complex responsible for regulating OPA1 proteolysis, thus regulating mitochondrial fusion. CLPB was found to interact with the PHB complex and with the mitochondrial antiviral signaling protein (MAVS) present at the outer mitochondrial membrane [8].

CLPB is upregulated in human acute myeloid leukemia (AML) cells [7]. The acquisition of resistance to the selective BCL2 inhibitor Venetoclax was linked to further CLPB upregulation, whereas ablation of CLPB re-sensitized AML cells [7] thus linking CLPB to highly specific regulatory processes of clinical significance. That study further demonstrated a direct interaction between CLPB and OPA1, and linked CLPB to proper cristae morphology. CLPB depletion constituted a pro-apoptotic factor by induction of the mitochondrial stress response and cristae remodeling. CLPB was further found in that study to directly interact with the HAX1 protein, another anti-apoptotic factor, as already postulated in our initial work [1].

Identification of the exact mechanism of CLPB action will be of importance to unravel the molecular basis of the CLPB defect. It will also shed light on other cellular regulatory processes in which CLPB is involved, including a possible role in apoptosis, oncogenesis and the innate viral response. Purification and initial biochemical characterization of human CLPB presented in this work will therefore not only enable future genotype-phenotype correlations, but may also pave the way towards deeper understanding of mitochondrial regulatory processes.

Contributions

S.Z. designed the study. S.Z., D.M., H.W., K.Z., T.S., performed the experiments. K.B. performed AFM imaging. R.D. performed CD spectroscopy and analyzed data. S.Z., R.W., S.W. analyzed the data and wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2020.129512.

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