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Differential degradation for small heat shock proteins IbpA and IbpB is synchronized in *Escherichia coli*: Implications for their functional cooperation in substrate refolding

Xiaodong Shi [a,b], Linxuan Yan [b], Hanlin Zhang [b], Kai Sun [a], Zengyi Chang [b,*], Xinmiao Fu [b,*]

[a] Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical College, Xuzhou, Jiangsu 221002, China
[b] State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing 100871, China

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ABSTRACT

Small heat shock proteins (sHSPs), as a conserved family of ATP-independent molecular chaperones, are known to bind non-native substrate proteins and facilitate the substrate refolding in cooperation with ATP-dependent chaperones (e.g., DnaK and ClpB). However, how different sHSPs function in coordination is poorly understood. Here we report that IbpA and IbpB, the two sHSPs of *Escherichia coli*, are coordinated by synchronizing their differential *in vivo* degradation. Whereas the individually expressed IbpA and IbpB are respectively degraded slowly and rapidly in cells cultured under both heat shock and normal conditions, their simultaneous expression leads to a synchronized degradation at a moderate rate. Apparently, such synchronization is linked to their hetero-oligomerization and cooperation in binding substrate proteins. In addition, truncation of the flexible N- and C-terminal tails dramatically suppresses the IbpB degradation, and somehow accelerates the IbpA degradation. In view of these *in vivo* data, we propose that the synchronized degradation for IbpA and IbpB are crucial for their synergistic promoting effect on DnaK/ClpB-mediated substrate refolding, conceivably via the formation of IbpA–IbpB-substrate complexes. This scenario may be common for different sHSPs that interact with each other in cells.

1. Introduction

Molecular chaperones and proteases play key roles in the *in vivo* proteostasis respectively by assisting in the correct folding/refolding of proteins and degrading misfolded ones [1,2]. Small heat shock proteins (sHSPs), as a conserved family of molecular chaperone, are characterized by a low molecular mass of 12–43 kDa, formation of large oligomers and exhibition of ATP-independent chaperone activity [3–11]. They are able to bind non-native substrate proteins and facilitate the substrate-refolding under the help of other ATP-dependent molecular chaperones (e.g., Hsp70/DnaK and Hsp100/ClpB) under both *in vitro* and *in vivo* conditions [12–21].

In general, high eukaryotic organisms encode more than ten sHSP genes while most prokaryotic cells contain only one or two sHSP genes [4,22]. This raises an interesting question over the biological significance, if any, of the presence of multiple members of the sHSP family in one organism or a cell. One possibility is that these different sHSPs work independently (i.e., with no additive or synergistic effects in chaperone functions). For instance, certain sHSPs from the bacterium *Deinococcus radiodurans* [23], the parasite *Toxoplasma gondii* [24] or plants [25] were reported to work in this way. Another possibility is that these sHSPs function in coordination with each other, given that different types of sHSPs from the same or closely related organisms were found to form hetero-oligomers [26–33]. In support of this, IbpA and IbpB, the sHSPs of *Escherichia coli* (*E. coli*) were reported to synergistically facilitate DnaK/ClpB-catalyzed substrate refolding [15,19–21].

We have been attempting to investigate the chaperone activity and mechanism of sHSPs under both *in vitro* [37–41] and *in vivo* conditions [42–44] for years. The present study is part of our efforts to understand the functional cooperation mechanism between different sHSPs of a cell. Here we examined the fate of sHSPs, instead of the substrates as widely undertaken [15,19–21], and found that the slow and fast degradation rates respectively for the individually expressed IbpA and IbpB were synchronized at an in-between degradation rate when they were co-expressed in cells. This considerably extends the earlier study [36], in which IbpA and IbpB were found to be degraded by the protease Lon

* Corresponding authors. Address: Room 204, School of Life Sciences, Peking University, Beijing 100871, China. Fax: +86 10 62751526.
E-mail addresses: changzy@pku.edu.cn (Z. Chang), fuxinmiao@pku.edu.cn (X. Fu).

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under *in vitro* conditions, respectively at slow and fast rates. The synchronized degradation may be a result or a prerequisite for the functional cooperation between IbpA and IbpB, conceivably through the formation of IbpA–IbpB-substrate complexes.

2. Material and methods

2.1. Bacterial strains

E. coli BW25113 wild type, Δ*ibpA* and Δ*ibpB* strains were obtained from Nara Institute of Science and Technology in Japan. *E. coli* MC4100-Δ*ibpAB* strain is a generous gift from Professor Baynex in Washington University in USA. *E. coli* DH5α cells were purchased from Transgen Company in Beijing.

2.2. Plasmid construction and protein expression

The DNA fragments containing *ibpA/B* or the truncation mutant genes were amplified by PCR from the genomic DNA of *E. coli* BW25113. The amplified DNA fragments were inserted into the pBAD expression vector after digested with NcoI and HindIII. The generated plasmids were then transformed into target strains for expressing the recombinant proteins under the induction of 0.02% arabinose.

2.3. Detection of protein degradation in cells

In vivo degradation was performed using the methods as reported earlier [36]. In brief, cells were grown at 37 °C in a water-bath shaker to OD_{600} of 0.6 and subjected to heat shock of 45 °C for 30 min. Spectinomycin was then added (at a final concentration of 400 μg/ml) to the cell cultures to stop protein translation. Cells were continuously incubated at 45 °C or 30 °C for varying length of time before lysed and analyzed by SDS–PAGE. Immunoblotting of IbpA and IbpB and their variants were performed using a polyclonal antibody against both IbpA and IbpB [43]. The TnaA protein, as an internal control for sample loading, was immunoblotted using a polyclonal antibody against TnaA (produced in our own laboratory [42]).

3. Results

3.1. The respective slow and fast in vivo degradation rates of IbpA and IbpB are synchronized when they are co-expressed in E. coli under both heat shock and normal conditions

In the earlier study [36], Bissonnette et al examined the Lon-mediated *in vitro* degradation of IbpA/IbpB and the *in vivo* degradation of IbpA at 37 °C. Here we probed the *in vivo* degradation of IbpA and IbpB in cells cultured at heat shock temperature (45 °C), given that both IbpA and IbpB were found to be hardly

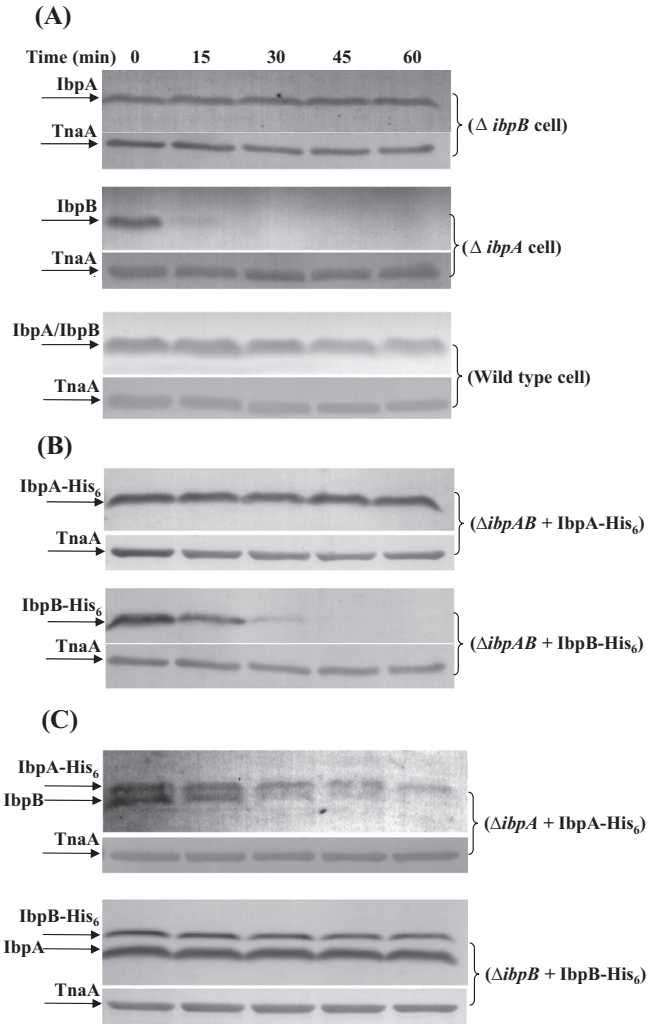

Fig. 1. Slow and fast *in vivo* degradation rates respectively for IbpA and IbpB were synchronized when they were co-expressed. Immunoblotting results of endogenously (Panel A) or exogenously (Panels B and C) expressed IbpA and IbpB in cells under indicated genetic backgrounds, using a polyclonal antibody against both IbpA and IbpB [43]. The cells, in which IbpA and IbpB were individually or simultaneously expressed, were heat shocked at 45 °C and maintained at 45 °C for indicated time before lysed and subjected to SDS–PAGE and immunoblotting analysis. TnaA was detected as an internal control for sample loading.

Table 1
Half-lives of IbpA/B and their variants in cells.[a]

Protein	Half-life (min)									
	When expressed alone					Co-expressed with				
	Intact[b]	His	ΔN11	ΔC11	ΔN11ΔC11	Intact[b]	His[c]	ΔN11[c]	ΔC11[c]	ΔN11ΔC11[c]
IbpA	NA/2214	2644	191	535	284	76/63	128/977	ND	ND	78/886
IbpB	24/24[d]	27	42	50	149		28/32	84/88	NA/560	113/231

[a] The half-life was calculated by analyzing the data presented in Fig. S2 using the linear decay model. NA: not available, reflecting the failure of linear decay regression analysis due to the extremely stability of the target proteins. ND, not determined, as a result of inability to separate IbpA from IbpB-N11 or IbpB-C11 on the SDS-gel due to their very close molecular sizes.

[b] Two values respectively represent the half-lives of IbpA/IbpB at 45 °C and 30 °C. The half-life (76 min) obtained here for the co-expressed IbpA/IbpB is apparently longer than that (15 min) as determined previously by Bissonnette et al. [36], presumably due to the difference in bacterial strains (note: the wild type strain used in our study is BW25113 while theirs was not mentioned).

[c] Two values respectively represent the half-lives of the target protein and of the co-expressed protein.

[d] The decay pattern of IbpB protein alone is apparently obeying the exponential decay model. In this context, the half-lives of IbpB alone at 45 °C and 30 °C would be 7.6 and 9.6 min, respectively.

detectable under normal conditions but substantially induced by heat shock [15,45]. Spectinomycin was added to the cell cultures to stop protein synthesis, which allowed us to chase the degradation of the pre-existed proteins. Data displayed in Fig. 1A indicate that the IbpA protein in Δ*ibpB* cells did not show significant degradation within 1 h (the upper panel), in contrast to the IbpB protein in Δ*ibpA* cells that was significantly degraded at the time point of 15 min (the middle panel). Notably, IbpA and IbpB as a whole were degraded at a moderate rate in wild type cells (the down panel of Fig. 1A).

To exclusively distinguish IbpA and IbpB, we attached a 6×His-tag at their C-terminals and individually expressed each as a recombinant protein in Δ*ibpAB*, Δ*ibpA* or Δ*ibpB* cells. Data presented in Fig. 1B indicate that in Δ*ibpAB* cells IbpA-His_6 did not show significant degradation while IbpB-His_6 was rapidly degraded, consistent with the properties of their intact forms (Fig. 1A). Again, the exogenously expressed IbpA-His_6 and endogenously present IbpB in Δ*ibpA* cells were found to be degraded in a largely synchronized manner at a moderate rate (the upper panel of Fig. 1C). Nevertheless, the exogenously expressed IbpB-His_6 and endogenously present IbpA in Δ*ibpB* cells did not show significant degradation (the down panel of Fig. 1C), presumably due to stabilization effect of the His-tag on the IbpB-His_6-IbpA hetero-oligomers. It should be pointed out that the protein level of recombinant IbpA-His_6 or IbpB-His_6 is comparable with that of endogenously expressed IbpA/IbpB (Fig. 1C), thus avoiding potential side-effect of protein over-expression. Together, the rapid degradation of IbpB is retarded in the presence of IbpA while the slow degradation of IbpA is accelerated in the presence of IbpB, resulting in the synchronization on their differential degradation rates.

We also examined whether the synchronized degradation of IbpA and IbpB, as observed at 45 °C (Fig. 1), would occur at normal temperatures. To this end, we kept *E. coli* cells expressing IbpA and/or IbpB at 45 °C for 30 min, added spectinomycin to stop protein synthesis, and then recovered the cells at 30 °C for varying length of time. Data presented in Fig. S1 demonstrate that the *in vivo* degradation rate at 30 °C of the individually expressed IbpB was much faster than that of IbpA, and such remarkably different degradations were synchronized when the two proteins were co-expressed, being similar with what took place at 45 °C (Fig. 1A).

We further performed semi-quantification analysis on the immunoblotting results and obtained the half-lives of IbpA and IbpB proteins (for details, see Fig. S2). Data presented in Table 1 indicate that, at both 45 °C and 30 °C, the half-life of the endogenous IbpA was >2000 min and IbpB around 24 min when they were individually expressed, and synchronized to 76 min when they were co-expressed. Notably, the half-lives of the individually expressed IbpA-His_6 and IbpB-His_6 were respectively comparable with those of the intact forms, and the co-expressed IbpB and IbpA-His_6 were synchronized at a half-life of around 30 min (Table 1).

3.2. The in vivo degradation rate of IbpB is significantly decreased by the deletion of its N- or C-terminal tails and is proportional to its chaperone activity

The N-terminal arm and C-terminal extension of sHSPs were widely reported to play crucial roles in the oligomerization and functional integrity of sHSPs [21,30,39–40,46–48], including the 11 residues at the N- and C-termini of IbpB as we reported earlier [40]. Here, the removal of these terminal tails was found to significantly suppress the degradation of IbpB (Fig. 2A), indicating their destabilization effect on IbpB. This is in line with the earlier report [36] that the purified full length IbpB protein was degraded by Lon at a 5-fold higher rate than did the truncated protein (i.e., the

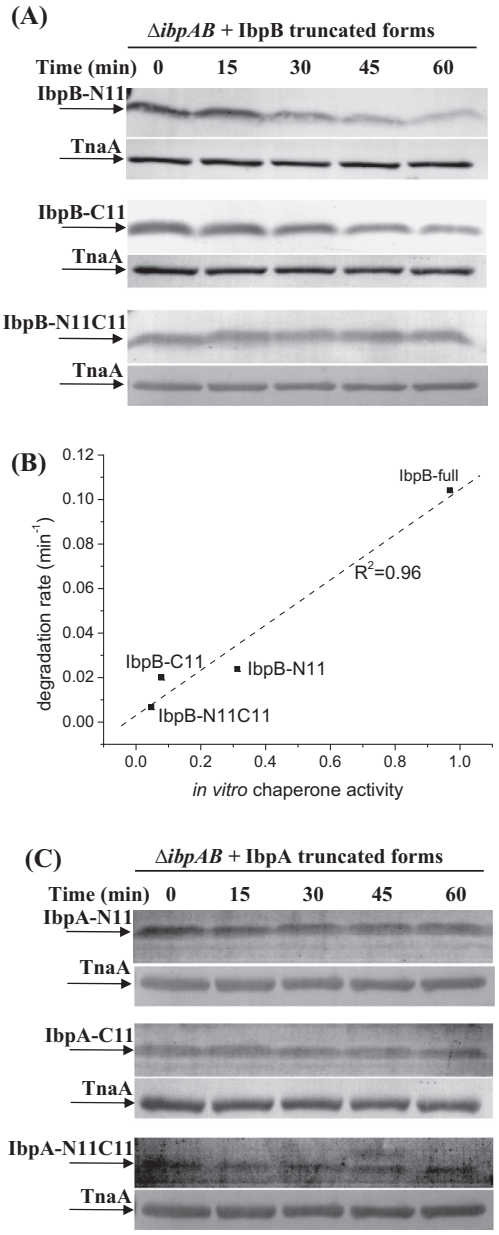

Fig. 2. Truncation of the N- and the C-terminal tail significantly suppressed the IbpB degradation while a similar truncation limitedly accelerated the IbpA degradation. (A) Immunoblotting results of truncated IbpB (Panel A) and IbpA (Panel C) proteins (i.e., lacking the N- and/or C-terminal 11 residues) that were exogenously expressed in the Δ*ibpAB* cells. Experimental conditions are similar with that as described in Fig. 1. (B) The degradation rate of IbpB is plotted against its *in vitro* chaperone activity, with a linear correlation being detected. The degradation rate for the full length and truncated IbpB was calculated as the reciprocal of their half-lives as shown in Table 1. The *in vitro* chaperone activity of each protein was calculated by measuring its capability in suppressing insulin aggregation, with the experimental data being adopted from our earlier report [40].

α-crystallin domain of IbpB). Specifically, the half-lives of IbpB-N11, IbpB-C11 and IbpB-N11C11 were 42, 50 and 149 min, respectively (Table 1), which were significantly lower than that of the intact form (24 min) and were comparable with that of IbpB (76 min) in the presence of IbpA.

Since these IbpB truncated proteins were all found to exist as dimers [40] but are substantially different in their half-lives, the half-life of IbpB appears not to be determined by its oligomerization state. Strikingly, we observed a significantly positive correlation for IbpB between the degradation rate (reciprocal to

the half-life) and the *in vitro* chaperone-like activity in suppressing the aggregation of reduced insulin B chain (R^2 = 0.96; Fig. 2B), indicating that the rapid *in vivo* degradation of the IbpB protein is closely linked to its chaperone function and substrate-binding.

3.3. The extremely slow in vivo degradation of IbpA is somehow accelerated by the deletion of its N- or C-terminal tails

IbpA and IbpB share 52.2% amino acid sequence identity [34] and the removal of 11 residues from the N- or the C-terminal tail of IbpA was recently observed to dramatically decrease its chaperone activity and impair the fibril formation [21]. Here we observed that the removal of the 11 residues for IbpA somehow accelerated its *in vivo* degradation (Fig. 2C), indicating their stabilization effect on IbpA. The half-lives of IbpA-N11, IbpA-C11 and IbpA-N11C11, being around 200–500 min (Table 1), is lower than that of the full length protein (>2000 min) but significantly longer than that of the full length IbpA protein in the presence of IbpB (76 min). In addition, we found that the truncated IbpA proteins were able to suppress IbpB degradation and that the IbpB-N11C11 variant protein was able to accelerate IbpA degradation (Fig. S3 and Table 1), indicating that the N- or C-terminal tails of IbpA and IbpB are not essential for the reciprocal effect on the degradation of the counterpart.

4. Discussion

It was reported earlier that the Lon-mediated *in vitro* degradation rates for IbpA and IbpB were respectively slow and fast, and that IbpB was able to facilitate the degradation of IbpA under both *in vitro* and *in vivo* conditions [36]. Our study reveals that the *in vivo* degradation rates for IbpA and IbpB are also respectively slow and fast. Importantly, the simultaneously expressed IbpA and IbpB synchronize their remarkably different degradations at a moderate rate, under both normal (Fig. S1) and heat shock growth conditions (Fig. 1).

The slow and fast Lon-mediated *in vitro* degradation rates respectively for IbpA and IbpB [36] apparently stems from the difference in their oligomeric structures, given that the purified IbpA and IbpB proteins were found to exist respectively as fibrils [21,49] and as large spherical oligomers (>2 MDa) [40,50]. It follows that the slow *in vivo* degradation for IbpA, as observed here, may also be due to its formation of fibrils under *in vivo* conditions [49]. In support of this, the truncated IbpA forms with slower degradation than the intact form (Fig. 2C) were reported to be less efficient than the intact form in forming fibrils under *in vitro* conditions [21]. In this context, the acceleration of IbpA degradation by IbpB is due to the loss of IbpA fibrils in the presence of IbpB and the subsequent formation of IbpA–IbpB spherical hetero-oligomers as reported earlier [19,21]. Interestingly, the *in vivo* degradation rate of IbpB in the absence of IbpA was found to be positively correlated with its *in vitro* chaperone activity (Fig. 2B), thus implicating that substrate-binding may promote the degradation of IbpB.

Our *in vivo* data, together with the earlier reported facts that Lon degrades substrate-bound IbpA/IbpB but not the substrate proteins under *in vitro* conditions [36] and that the substrate refolding from the IbpA–IbpB-substrate complexes is efficiently facilitated by DnaK/ClpB [14–15,19–21], indicate a dual-process scenario of IbpA/IbpB-degradation and substrate-refolding in cells, where these two processes cross-talk with each other. Further, we propose that the synchronized IbpA/IbpB degradation may play crucial roles for their known functional cooperation in substrate-refolding (as illustrated in Fig. 3).

First, the individually expressed IbpA, existing as IbpA fibrils and/or IbpA-substrate complexes, is degraded very slowly (left part in Fig. 3). Second, the individually expressed IbpB, existing as IbpB oligomers and/or IbpB-substrate complexes, is degraded very rapidly (left part in Fig. 3). However, the slow degradation of IbpA in the IbpA-substrate complexes and the rapid degradation of IbpB in the IbpB-substrate complexes may lead the substrates to be released too slowly and too rapidly, respectively, both of which are not optimal for the subsequent delivery of substrates to the DnaK–ClpB chaperone system. As a result, inefficient substrate refolding would occur as observed earlier [19–21]. Third, when they are co-expressed as naturally taking place in wild type cells, IbpA and IbpB form IbpA–IbpB-substrate complexes and/or IbpA–IbpB hetero-oligomers, and would be degraded in a synchronized manner at a moderate rate (right part in Fig. 3). Conceivably, this would allow the substrates to be released from the IbpA–IbpB-substrate complexes at an appropriate rate, which is optimal for the loading and refolding capacity of the DnaK–ClpB chaperone system [19–21]. The synchronization in protein degradation for IbpA and IbpB is in agreement with their simultaneous expression [34,35,45,51–52], co-localization in protein aggregates [34,52–53] and hetero-oligomerization [19,21], all of which appear to be

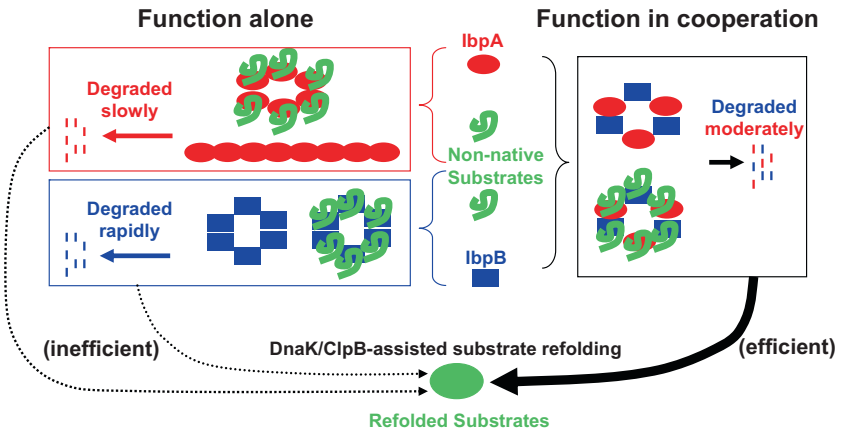

Fig. 3. Schematic illustration of the synchronized IbpA/IbpB degradation mechanism underlying their functional cooperation for substrate refolding. The individually expressed IbpA, existing as fibrils and/or as IbpA-substrate complexes, is degraded very slowly such that the substrates cannot be effectively released. By contrast, the individually expressed IbpB, existing as IbpB-substrate complexes and/or as self-oligomers, is rapidly degraded, accompanied with a rapid release of substrates. The co-expressed IbpA and IbpB, existing as IbpA–IbpB-substrate complexes and/or as IbpA–IbpB hetero-oligomers, are degraded in a synchronized manner at a moderate rate, which, in turn, allows the substrates to be released at an appropriate rate for efficient refolding under the assistance of the DnaK–ClpB chaperone system.

linked to their synergistic promoting effect on the DnaK/ClpB-catalyzed substrate refolding [19–21].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.084.

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