The GTPase BipA expressed at low temperature in *Escherichia coli* assists ribosome assembly and has chaperone-like activity

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BPI-inducible protein A (BipA) is a conserved ribosome-associated GTPase in bacteria that is structurally similar to other GTPases associated with protein translation, including IF2, EF-Tu, and EF-G. Its binding site on the ribosome appears to overlap those of these translational GTPases. Mutations in the *bipA* gene cause a variety of phenotypes, including cold and antibiotics sensitivities and decreased pathogenicity, implying that BipA may participate in diverse cellular processes by regulating translation. According to recent studies, a *bipA*-deletion strain of *Escherichia coli* displays a ribosome assembly defect at low temperature, suggesting that BipA might be involved in ribosome assembly. To further investigate BipA’s role in ribosome biogenesis, here, we compared and analyzed the ribosomal protein compositions of MG1655 WT and *bipA*-deletion strains at 20 °C. A aberrant 50S ribosomal subunits (i.e. 44S particles) accumulated in the *bipA*-deletion strain at 20 °C, and the ribosomal protein L6 was absent in these 44S particles. Furthermore, *bipA* expression was significantly stimulated at 20 °C, suggesting that it encodes a cold shock-inducible GTPase. Moreover, the transcriptional regulator CAMP receptor protein (CRP) positively promoted *bipA* expression only at 20 °C. Importantly, GFP and α-glucosidase refolding assays revealed that BipA has chaperone activity. Our findings indicate that BipA is a cold shock-inducible GTPase that participates in 50S ribosomal subunit assembly by incorporating the L6 ribosomal protein into the 44S particle during the assembly.

GTPases are one of the most widely distributed protein families in all kingdoms of life and play pivotal regulatory roles in various cellular processes. Largely, the GTPase superclass can be divided into two classes, the TRAFAC (translation factors) class and the SIMIBI (signal recognition particle, MinD, and BioD) class. The TRAFAC class can also be divided into two subgroups, translational GTPases (trGTPases) and nontranslational GTPases. The former includes EF-G, EF-Tu, IF2, and LepA and participates in facilitating protein synthesis or controlling translation fidelity. By contrast, MnmE, Era, Der, YihA, and ObgE belong to nontranslational GTPases and participate in signal transduction, cell motility, and ribosome assembly (1).

BipA (BPI-inducible protein A), also known as TypA (tyrosine phosphoprotein A), is a trGTPase based on its primary sequence and structural similarities. The *bipA* gene is highly conserved in a variety of bacteria and is also detected in chloroplast genomes (2, 3). Like EF-G and LepA, BipA interacts with the ribosome and is composed of five domains, including the N-terminal G-domain, domain II, domain III, domain V, and unique C-terminal domain (4). The binding site of BipA is close to the peptidyltransferase center of the ribosome to which the other trGTPases bind. Structural studies have revealed that BipA forms multiple contacts with the ribosome; the G-domain of BipA binds to ribosomal protein L12 of the 50S ribosomal subunit, domain II to helices 5 and 15 of 16S rRNA, domain III to ribosomal protein S12 of the 30S ribosomal subunit, domain V to L11 of the 50S ribosomal subunit, and the C-terminal domain to helix 89 of 23S rRNA. In addition to the ribosome components, BipA also binds to tRNA located in the A-site of the ribosome (4).

In *Salmonella enterica* serovar Typhimurium, BipA exhibits two ribosome-binding modes, which depend on the type of nucleotide bound to BipA (6). In vivo, BipA associates with the 70S ribosome even without nucleotides under optimal growth conditions; however, in vitro exogenous addition of GTP was required for the 70S ribosome association. Moreover, under stress conditions, such as an increased guanosine 3′,5′-bispyrophosphate (ppGpp) level and high and low temperatures, BipA binds to the 30S ribosomal subunit both *in vivo* and *in vitro* (7–9). These results suggest that BipA may be a stress response regulator by modulating its ribosome association depending on the nucleotide.

Although the *bipA* gene is highly conserved in bacteria, its deletion causes no significant phenotype in *Escherichia coli* under optimal growth conditions. Conversely, under stress conditions, specifically low temperature or nutrient depletion, BipA becomes essential for growth in *E. coli* (10, 11). In *Pseudomonas putida* and *Bacillus subtilis*, BipA is also crucial for...
survival at low temperature (12, 13). In addition, in *Sinorhizobium meliloti*, BipA is indispensable under not only low-temperature but also low-pH conditions and in the presence of SDS (14). These findings provide clues that BipA is involved in virulence as well as the stress response. In *Salmonella Typhimurium*, expression of BipA is induced by BPI (Bactericidal/permeability-increasing protein), an antimicrobial peptide produced by neutrophils and part of the innate immune system (15–17). Furthermore, BipA is implicated in regulating various cellular processes, including antimicrobial resistance, motility, capsule production, and biofilm formation (18–20).

Despite its binding to the 70S ribosome and 30S ribosomal subunits, interestingly, several study findings support the hypothesis that BipA may be involved in 50S ribosomal subunit assembly. At low temperature, a *bipA*-deletion strain showed an abnormal ribosome profile, in which the 30S ribosomal subunit was accumulated and pre-50S particles were observed. Furthermore, neither 23S rRNA nor 16S rRNA were properly processed, suggesting that 50S ribosomal rather than 30S ribosomal subunit synthesis was more severely influenced (21).

Although there have been many studies, the cause of the cold-sensitive phenotype by deletion of *bipA* and the role played by BipA at low temperature remain elusive. Therefore, in this study, we investigated the ribosome assembly defect caused by deletion of *bipA* in more detail and elucidated the precise role and mechanism of BipA under low-temperature conditions.

**Results**

**Deletion of bipA causes accumulation of pre-50S particles lacking ribosomal protein L6**

Deletion of *E. coli* *bipA* led to an accumulation of unprocessed 23S and 16S rRNAs with the concomitant appearance of abnormal 50S ribosomal subunit particles at low temperature (21); however, it remains ambiguous what role BipA plays in ribosome assembly and how BipA modulates its ribosome association upon nucleotide binding. To clarify these questions, sucrose gradient sedimentation was performed using cells cultured at 37 or 20 °C. We had previously confirmed the cold-sensitive growth defect of the *bipA*-deletion strain (ESC19), as shown in Fig. S1. ESC19 cells normally formed colonies at both 37 and 30 °C, but at 20 °C, *bipA*-deleted cells showed growth defects on the Luria–Bertani (LB) plate. To investigate the abnormality of the ribosome profile of ESC19 cells, MG1655 and ESC19 cells were cultured in LB medium at 37 or 20 °C, and the cultures were withdrawn for polysome and subunit profile analyses. Then the cleared cell lysates were subjected to sucrose density gradient fractionation. As shown in Fig. 1A, there were no significant differences in polysome profiles between
Chaperone-like activity of BipA

MG1655 and ESC19 grown at 37 °C. However, in ESC19 cells grown at 20 °C, the 30S ribosome subunits were accumulated out of proportion to the 50S ribosomal subunits, and an unusual peak appeared between the 50S and 30S subunit positions, implying an unstable feature of the 50S ribosomal subunit. To further examine ribosome assembly in strain ESC19, subunit profile analysis was performed. At low temperature, bipA deletion caused a reduction of the 50S ribosome subunits and an accumulation of aberrant ribosomal subunits (~44S particles), which were observed between the 50S and 30S ribosomal subunit peaks at 20 °C (Fig. 1B). Thus, considering the accumulation of rRNA precursors in bipA-deleted cells, it is likely that the lack of BipA inhibited 50S ribosome assembly, resulting in the appearance of the 44S particles.

Next, to investigate the nature of the accumulated 44S particles in ESC19 at low temperature, we compared the ribosomal protein composition of ESC19 with that of MG1655. The sucrose gradient fractions containing 50S ribosomal subunits, 44S particles, or 30S ribosomal subunits were combined, and each ribosomal subunit sample was analyzed by SDS-PAGE and silver staining. Compared with the 50S ribosomal subunit of MG1655 cells, one ribosomal protein was largely missing in the 44S particles, and it was identified as ribosomal protein L6 by MS analysis (Fig. 1C). In addition, ribosomal proteins L9 and L18 were underrepresented. By contrast, the ribosomal protein compositions of the 30S ribosomal subunit were not affected by bipA deletion (Fig. 1C). L6 ribosomal protein interacts with 23S rRNA and is assembled into the 50S ribosomal subunit during a late step in 50S ribosomal subunit biogenesis (22, 23). Interestingly, the L6 mutant strain also shows defects in growth and ribosome assembly at low temperature (24, 25). These results suggest that BipA may participate in incorporating L6 into the 50S ribosomal subunit at low temperature and that the cold sensitivity of ESC19 was caused by accumulation of 44S particles lacking L6 ribosomal proteins.

Ribosome binding of BipA is regulated by nucleotides

To determine how endogenous BipA modulates its association with the ribosome depending on its nucleotide-bound status, we investigated BipA localization in the presence of nucleotides. We cultured MG1655 cells in LB medium at 37 or 20 °C until an A600 of 0.5–0.6 was obtained, after which cells were lysed in buffer BP supplemented with 100 μM nucleotides, such as GMPPNP (nonhydrolyzable analog of GTP), GDP, or ppGpp. The samples were subjected to sucrose gradient sedimentation analysis followed by Western blotting using anti-BipA antibodies. When cells were cultured at 37 °C, most BipA proteins were located in soluble fractions in the absence of nucleotides (Fig. 2). Similarly, GDP did not promote the association of BipA with the ribosome.

To investigate the effect of increased intracellular ppGpp levels on the localization of BipA, MG1655 cells were either transformed with pRelA or treated with serine hydroxamate (SHX). SHX induces amino acid starvation by inhibiting the charging of seryl-tRNA synthetase, thereby leading to an accumulation of ppGpp (26). The exogenous expression of the ppGpp-synthesizing enzyme (RelA) or stringent response induction by SHX did not induce the association of BipA with the ribosome. However, in the presence of GMPPNP, BipA proteins were detected in the 30S and 50S ribosomal subunits, 70S ribosome, and polysome fractions. This ribosome-binding mode was similar at both 20 and 37 °C, although more BipA proteins were detected at 20 °C. It should be noted that the ribosome-binding mode of Salmonella Typhimurium BipA changed from the 70S ribosome to the 30S ribosomal subunit in response to ppGpp (6).

GTPase activity is essential for BipA to facilitate ribosome assembly

To further demonstrate the GTP-dependent binding of BipA to the ribosome, we constructed mutant clones pACYC184BipAN128D and pET28BipAN128D expressing BipAN128D whose mutation is located at the conserved G4 motif (NKXD) and hampers hydrogen bond formation with the 2-amino group of the guanine ring (27).

Prior to the in vivo investigation, we confirmed that GTPase activity was effectively inhibited by the mutation of Asn-128 to Asp. His6-tagged WT and mutant BipA proteins were overex-
pressed and purified, and the GTPase assay was performed as described under “Materials and methods.” The results revealed that the Asn-128 to Asp substitution disrupted GTP hydrolysis activity (Fig. S2).

To investigate whether the expression of BipAN128D could recover the cold sensitivity of strain ESC19, ESC19 cells were transformed with pACYC184, pACYC184BipA, or pACYC184BipAN128D. Transformants were incubated at 20 °C for 48 h in LB medium containing kanamycin and chloramphenicol. As shown in Fig. 3A, BipAN128D could not support the growth of ESC19 cells at 20 °C. Polysome and subunit profile analysis experiments showed that the mutation in the Figure 3. Phenotypes of the ESC19 cells expressing BipAN128D. A, growth curves of ESC19 cells carrying pACYC184, pACYC184BipA, or pACYC184BipAN128D. Cells were cultured in LB medium at 20 °C, and at each time point, the cultures were diluted 5 times before measurement of the optical density at 600 nm. Each value was averaged from two independent experiments, and error bars represent S.D. Polysome (B) and subunit (C) profiles of ESC19 expressing WT or BipAN128D are shown. Sucrose density gradient sedimentation was performed as in Fig. 1. Polysome samples were prepared in the presence of GMPPNP. Arrows, aberrant 44S particles. D, SDS-PAGE analysis of 50S and 44S ribosomal subunits from ESC19 expressing BipAN128D. Composition of ribosomal proteins of ribosomal subunits was analyzed as described in the legend to Fig. 1. Experiments were performed in two independent repetitions, and data shown are representative results. M, molecular weight marker.

E, inability of BipAN128D to interact with ribosome. Western blot analysis was carried out using the fractions from B. Experiments were performed in two independent repetitions, and data shown are representative results. M, molecular weight marker.
Asn-128 residue resulted in ribosome abnormality (Fig. 3, B and C). Unlike WT BipA, the expression of BipAN128D in ESC19 cells led to the significant reduction of 50S ribosomal subunits and the accumulation of 44S particles. The amount of 70S ribosomes was slightly reduced, and that of free 30S ribosomal subunits was increased, consistent with Fig. 1. The 44S particles isolated from ESC19 cells harboring pACYC184BipAN128D did not contain the L6 protein (Fig. 3D). In addition, BipAN128D lost its ability to associate with 70S, 50S, 44S, or 30S ribosome particles (Fig. 3E). Taken together, our results indicated that GTP was required for ribosome association and that BipA is directly involved in 50S ribosome assembly.

**Expression of bipA is stimulated by low temperature**

Although BipA is not essential for growth under optimal conditions, it is required under various stress conditions, such as cold shock (Fig. S1) (10, 11). As mentioned above, BipA in Salmonella Typhimurium is induced by BPI with antimicrobial activity against Gram-negative bacteria (15, 28). We hypothesized that BipA may be induced by low temperature. To determine whether the expression level of bipA increased at low temperature, strain MG1655 was grown at 37 °C for 3 h, followed by incubation at 20 °C for 30 min. Total RNA samples were prepared from cells harvested at 0 and 30 min after the temperature downshift. Next, the mRNA level of bipA was examined by qRT-PCR using rrsA as reference gene. The mRNA level of bipA increased ~25.6-fold at 20 °C compared with 37 °C (Fig. 4A). As a positive control, we analyzed the transcript of a representative major cold-shock gene, cspA, and it showed a dramatic increase after the temperature downshift (~33.2-fold) (29). However, unlike at 20 °C, heat stress did not influence the transcription of bipA (Fig. 4B). These results unambiguously demonstrate that, similar to that of cspA, the transcription of bipA is triggered by cold shock.

The binding of BipA to the ribosome in Salmonella Typhimurium switches from the 70S ribosome to the 30S ribosomal subunit in the presence of ppGpp (6), implying an involvement of subunit biogenesis. Ribosome biogenesis is negatively regulated under a stringent response (30). Therefore, we investigated whether ppGpp also modulates the transcription of bipA. To increase ppGpp levels in the cell, MG1655 cells were transformed with pACYC177 or pRelA (31) and collected after incubation at 37 °C until they reached an A600 of ~0.6. The transcription pattern of bipA was then monitored in the presence and absence of the exogenously expressed RelA protein. We also treated MG1655 cells with SHX as described under “Materials and methods” to increase intracellular ppGpp levels and monitored the change in bipA mRNA. Unlike in the low-temperature conditions, transcription of bipA was not stimulated by higher ppGpp levels (Fig. 4, C and D).

We conducted Western blot analysis to confirm the inducibility of bipA at low temperature. Cell cultures were prepared...
for Western blotting as shown in Fig. 4 (A and B) and harvested at various time points after the temperature down- or upshift. Furthermore, to examine the effect of high ppGpp levels on the expression of BipA, we grew MG1655 cells harboring pACYC177 or pRelA as described in Fig. 4 C or treated MG1655 cells with SHX as shown in Fig. 4 D, after which the cells were harvested. Total protein samples were subjected to SDS-PAGE followed by Western blot analysis using an anti-BipA antibody. After incubation at 20 °C for up to 4 h (Fig. 4E), the amount of BipA gradually increased (~3.5-fold). High temperature and ppGpp did not trigger the induction of BipA (Fig. 4, F–H). It should be noted that translational induction of BipA is less than its transcriptional induction, and this is likely due to the lack of translation machinery or a blocked translation initiation step at low temperature (32). Our findings indicate that BipA is a cold-inducible protein, the absence of which may cause defects in growth and ribosome assembly as observed in ESC19 under low-temperature conditions. Basal expression of BipA was also observed at 37 °C (Fig. 2).

Expression of bipA is positively regulated by cAMP receptor protein (CRP) at low temperature

To investigate the regulation of the expression of bipA at low-temperature conditions, we analyzed DNA sequences of the upstream region of bipA using bacterial promoter prediction programs and identified two possible CRP-binding sites, namely S1 (a highly conserved CRP-binding site) and S2 (a less conserved putative site) (Fig. 5 C). CRP is a global transcription-regulatory protein that is involved in sugar metabolism (33–35). cAMP binds CRP and induces its conformational change that allows CRP to associate with the target DNA region located in the promoter (36, 37). CRP regulates transcription through protein–protein interactions with RNA polymerase (38, 39). CRP controls the transcription of more than 300 genes and participates in a variety of cellular processes, such as iron uptake, persistence, biofilm formation, osmoregulation, and carbon metabolism (40–44). CRP also indirectly regulates the expression of cspA, cspB, cspE and cspI, which are known to be cold shock–
inducible proteins among the nine CspA family cold-shock proteins (45).

We examined whether CRP influences the cold-inducible expression of *bipa*. To compare the expression levels of *bipa* between WT and *crp*-deletion strains at low temperature, we first replaced the *crp* gene of the MG1655 strain with a kanamycin-resistance cassette, yielding the *crp*-deletion strain (ESC25). MG1655 and ESC25 cells were incubated, and the expression levels of *bipa* were measured as described under “Materials and methods.” Their relative expression levels were quantified based on the expression level of *bipa* of MG1655 at 37 °C. In the MG1655 strain, the transcription level of *bipa* increased ~26.4-fold in response to cold shock (Fig. 5A). In the strain ESC25, the *bipa* transcript at 37 °C was elevated 6.8-fold compared with that in the strain MG1655 at the same temperature. In contrast to the MG1655 strain, the *bipa* mRNA level in the ESC25 strain was not enhanced by cold shock (~7.9-fold) and was much lower than in MG1655 at 20 °C. These results suggest that CRP stimulates the expression of *bipa* as a transcriptional activator at low temperature but not at 37 °C.

Next, changes in the translational expression level of *bipa* were analyzed by Western blotting. After MG1655 and ESC25 cell cultures were incubated at 37 °C for 3 h and moved to 20 °C, the amount of BipA in MG1655 gradually increased and reached higher levels (3.3-fold) after 2 h, whereas the amount of BipA in ESC25 remained constant after the temperature downshift (Fig. 5B). These findings demonstrate that the cold-shock inducibility of *bipa* therefore depends on CRP. In addition, the strain ESC25 showed a growth defect at 20 °C (Fig. S3), supporting the importance of CRP under low-temperature conditions.

To further ascertain the direct involvement of CRP in the expression of *bipa*, we performed electrophoretic motility shift assays (EMSAs). As shown in Fig. 5C, S1 (a highly conserved consensus sequence) is located −96 to −81 bases upstream from the predicted transcription start site (denoted as +1), and S2 (a putative binding site) is located +9 to +16 bases downstream from the transcription starting site. Using DNA fragments containing S1, S2, or both, we examined the interaction between purified CRP-His6 and three different DNA fragments with native PAGE. CRP was able to bind to the BCBS I (−125 to +45) DNA fragment and BCBS II (−125 to −55) but not to BCBS III (−16 to +45), suggesting that the S1 site is a CRP-binding site (Fig. 5D).

To ascertain the specific binding of CRP to this CRP-binding site, we substituted the bases in the CRP-binding site (Fig. 5E) and performed EMSAs with CRP and WT or four mutated BCBS II DNA fragments in the presence of cAMP. None of the mutant BCBS II fragments formed a complex with CRP, indicating a specific recognition by CRP (Fig. 5F). In the absence of cAMP, this association was not maintained, suggesting that CRP binds in a cAMP-dependent manner (Fig. S4). Taken together, our results demonstrate that transcriptional stimulation of *bipa* is directly mediated by CRP at low temperature.

**BipA has a chaperone-like activity in its G-domain**

BipA homologs share a structural similarity with trGTPases, such as IF2, EF-Tu, EF-G, LepA, and RF3 (46). These trGTPases possess a protein chaperone activity in their G-domains (47–49). Furthermore, IF2 belongs to a group of cold shock–inducible proteins (50). Therefore, we hypothesized that BipA functions as a chaperone at low temperature and possibly during the process of ribosome assembly. To test this idea, we chose two proteins as substrates, namely GFP and α-glucosidase. The refolding of the denatured protein was investigated by measuring the activities of proteins after incubation with BipA as described under “Materials and methods.” The fluorescence of acid-denatured GFP decreased to 40% at 0 min compared with native GFP (Fig. 6A). When denatured GFP spontaneously refolded even without the chaperone protein, 67% of the fluorescence from the denatured GFP was recovered at 20 min. Incubation of denatured GFP with DnaK reactivated its fluorescence up to 76%. Similar to the presence of DnaK, that of BipA in the refolding reaction increased the percentage of refolded GFP to 86%.

To ensure that refolding of the denatured GFP was triggered by the chaperone activity of BipA, we performed GFP refolding in the presence of RNase A. The fluorescence activity of refolded GFP after incubation with RNase A was almost the same as that in the absence of a chaperone, suggesting that RNase A has no chaperone activity and that the refolding of denatured GFP was caused by the chaperone activity of BipA. As the concentration of BipA increased, the amount of refolded GFP also rose (Fig. 6C). Consistent results were obtained in refolding assays using α-glucosidase as a substrate (Fig. 6, B and D). Urea-denatured α-glucosidase was spontaneously recovered up to 28%. After incubation with BipA, denatured α-glucosidase was reactivated to 43%, similar to the percentage of α-glucosidase refolded by DnaK. As the concentration of BipA increased, the percentage of reactivated α-glucosidase increased. These results demonstrate that refolding of denatured proteins was induced by the chaperone activity of BipA.

The G-domains of EF-G and LepA, which are structurally similar to that of BipA, are essential to the proteins’ chaperone activity (49). To determine the importance of the G-domain of BipA in its chaperone activity, we constructed BipA variants by truncating segmented domains from the N-terminal or C-terminal end and purified them (Fig. 7A). The truncated protein, which contained only the G-domain, was unstable and was expressed in insoluble form, and therefore we could not purify it. Using GFP and α-glucosidase as the substrate, we measured the chaperone activities of these derivatives. Incubation with BipA, BipA(1–303), BipA(1–390), and BipA(1–480) mutant proteins increased the percentage of refolded GFP to 81, 84, 86, and 76%, respectively (Fig. 7B). Consistently, BipA, BipA(1–303), BipA(1–390), and BipA(1–480) mutant proteins promoted refolding of the denatured α-glucosidase at 55, 58, 58, and 57%, respectively (Fig. 7C). All C-terminal truncated BipA proteins exhibited chaperone activity equivalent to that of WT BipA. In contrast, after incubation with BipA(49–607) or BipA(149–607), the fluorescence activity of refolded GFP was almost the same as that in the absence of a chaperone (Fig. 7D). These mutants also did not promote the refolding of the denatured α-glucosidase (Fig. 7E). These results demonstrate that the chaperone activity is located in the G-domain in BipA, although we cannot exclude the possibility that the truncated proteins are misfolded.
Because the GTP-binding domain is responsible for nucleotide hydrolysis and switching activity, we measured the chaperone activity of BipA in the presence of various nucleotides: GDP, GTP, and GMPPNP (Fig. 7, F and G). Unlike the ribosome association, the types of nucleotide had no influence on chaperone activity. In the apo-state of BipA, 80% of the denatured GFP was refolded, and in the presence of GDP, GTP, and GMPPNP, 82, 84, and 82% of GFP was refolded, respectively. In the H9251-glucosidase–refolding assay, incubation with BipA and nucleotides increased the percentage of refolded proteins to 40–43%, which did not differ from that of refolded proteins after adding BipA (42%). We further demonstrated the nucleotide-independent chaperone activity of BipA using His6-tagged BipAN128D. Incubation with BipA or BipAN128D proteins increased the percentage of refolded GFP to 94 or 86%, respectively (Fig. 7H). Consistent results were obtained in the α-glucosidase–refolding assay. Following incubation with BipAN128D, denatured α-glucosidase was reactivated to 43%, similar to the percentage of α-glucosidase refolded by WT BipA (45%). This indicates that mutation in the GTP-binding sites did not affect the chaperone activity of BipA (Fig. 7I). Our results demonstrated that BipA has a nucleotide-independent chaperone activity, which is mediated by the G-domain but does not require either GTP binding or hydrolysis.

Discussion

In this study, we explored the possible role of BipA in ribosome biogenesis and demonstrated, for the first time, that BipA is a novel ribosome assembly GTPase with chaperone activity in its G-domain. We analyzed the composition of ribosomal proteins of particles that accumulated in the bipA-deletion strain at 20 °C and found that these particles were precursors of the 50S ribosomal subunit mostly lacking the L6 ribosomal protein and, to a reduced extent, the L9 and L18 proteins (Fig. 1C). In E. coli, rplF encoding the ribosomal protein L6 is essential, and its depletion caused an inability to form colonies and disrupted ribosome assembly (25). Processing of rRNA in L6-depleted cells remained intact, which indicated that L6 was incorporated into the 50S ribosomal subunit during the late stage of the assembly process. Nevertheless, L6-depleted cells accumulated immature 45S particles, which are generally defined as late-stage precursors in the assembly of the 50S ribosomal subunit (22, 25, 30). Considering the incomplete RNA processing and lack of L6 in the 50S ribosomal subunit of bipA-deleted cells (Fig. 1C), it is likely that BipA associates with the ribosome.
prior to L6 and promotes the incorporation of L6 into the 50S ribosomal subunit. In *B. subtilis*, RbgA (a nontranslational small GTPase that is not conserved in *E. coli*) enables the binding of L6 at the proper position in the 50S ribosomal subunit (51). Both BipA and LepA bind in a similar manner to the A-site of the 50S ribosomal subunit. BipA is located in proximity to L6 (Fig. S5) (52), and LepA not only makes direct contact with L6, but also incorporates L10 and L12 ribosomal proteins via its nucleotide-independent chaperone activity (46, 53). More recently, *lepA* strain was cold-sensitive and shown to accumulate the precursor 17S rRNA and 30S particles missing several ribosomal proteins, suggesting a novel 30S ribosome assembly GTPase (54). Similar to BipA and LepA, other trGTPases, such as IF2, EF-Tu, and EF-G, commonly possess molecular chaperone activity in their G-domains (47, 49). Our results strengthen previous findings that the G-domain, rather than its catalytic activity, is responsible for the chaperone activity (Figs. 6 and 7). Thus, based on our investigations, it appears that incorporation of L6 into ribosome depends on the GTPase activity of BipA; however, whether or not its chaperone activity may assist proper folding of L6 on ribosome and/or association with local ribosomal components remains to be investigated.

The levels of EF-Tu in cells were much higher than those of other protein synthesis machinery components, and therefore it was predicted that EF-Tu has an auxiliary function other than as a translation factor (48). Among trGTPases, only IF2 was found to be a cold shock–inducible protein (55). Similar to IF2, BipA may replenish chaperone activity in cold-stressed cells to resume ribosome assembly.

Although the nucleotide type does not matter for chaperone activity, it is important for BipA to associate with the ribosome. In the case of *Salmonella* Typhimurium, ribosome association of BipA was demonstrated *in vitro* by using purified His-tagged BipA proteins and ribosome particles, which showed that it binds to 70S in the presence of GTP and to 30S in the presence of ppGpp (6). Our findings demonstrated that endogenous BipA in *E. coli* K-12 binds to the 50S and 30S ribosomal subunits as well as the 70S ribosome in the presence of GMP-PNP (Fig. 2). However, ppGpp did not promote the interaction of BipA with ribosome and ribosomal subunits. Under stress conditions, such as nutrient deficiency and low/high-temperature conditions, GTP shows a substantial deficit, and ppGpp is responsively accumulated (56). ppGpp not only regulates the transcription process; it also affects the translation process by

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**Figure 7. The G-domain of BipA is responsible for chaperone activity.**

A. Schematic presentation of BipA variants. The chaperone activity of full-length and truncated BipA proteins was monitored using GFP (B and D) and α-glucosidase (C and E). The chaperone activity in the presence of nucleotides (100 μM) was measured with GFP (F) and α-glucosidase (G) as substrates. The chaperone activity of WT and mutant BipA proteins were monitored using GFP (H) and α-glucosidase (I). Three independent refolding assays were performed in the same manner as described in Fig. 6. Individual data points are represented, with error bars denoting S.D. The data were analyzed using the unpaired two-tailed *t* test. NS, nonsignificant; *, *p < 0.05; **, *p < 0.01; ***, *p < 0.001.
binding to IF2, EF-Tu, or EF-G (57–62). In the case of IF2, its affinity to ppGpp is higher than that to GTP and GDP, and IF2 preferentially binds to ppGpp, causing conformational changes. Eventually, the function of IF2 is inhibited (63). Furthermore, a large number of monosomes are accumulated during the stringent response, resulting from inhibition of IF2 and EF-G during the translation process by ppGpp (64, 65). Taken together, it is likely that in response to lower GTP concentrations, increased ppGpp may become an alternative substrate for BipA, which in turn disturbs the interaction of BipA with the ribosome. Based on these observations, we can speculate that under ppGpp-producing conditions, BipA is unable to interact with the ribosome, consequently reducing overall ribosome biogenesis.

As shown in Fig. 4, transcriptional expression of bipA at 20 °C was increased by 25.6-fold compared with 37 °C, as further confirmed by Western blot analysis, whereas it was not affected by high temperature or ppGpp level. It is noteworthy that the expression of bipA is induced up to 7-fold by antimicrobial peptide BPI in Salmonella Typhimurium (15).

This cold inducibility was mediated by CRP, whose consensus binding site was found in the promoter region of bipA (Fig. 5). CRP, which is known as a global regulator of sugar metabolism, also plays a detrimental role in cold adaption. Its deletion exerts growth defects at low temperature (66). Recently, the expression of cspA family genes, such as cspA, cspB, cspG, and cspL, was found to be indirectly up-regulated at 15 °C without binding of CRP to the promoter regions of those genes, and CRP binding to the promoter of cspE, an early cold-inducible gene, directly up-regulates its expression (45, 66, 67). Our results unambiguously indicate that bipA is overexpressed by CRP in a direct manner at low temperatures. However, although CRP-cAMP was suggested to participate in a variety of cellular functions, such as virulence, flagellum synthesis, biofilm formation, quorum sensing, and nitrogen regulation (44, 68–72), it is unknown whether CRP and/or cAMP are also cold-inducible under the conditions we tested. Thus, we are currently investigating this question.

In addition to defects in growth and ribosome assembly in ESC19 cells at low temperature, BipA is also implicated in flagella-mediated cell motility and capsule synthesis at low temperature (19, 20). Like EPEC, in MG1655, the production of capsule was significantly increased by bipA deletion at 20 °C (~20.8-fold) (Fig. S6A), suggesting that BipA may be involved in the negative regulation of capsule synthesis at low temperature. By contrast, the results of the motility assay were different between EPEC and MG1655. In EPEC, the bipA null mutant was hypermotile at 37 °C on motility plates, but ESC19 (bipA deletion in E. coli K-12) was less motile than WT at both 37 and 20 °C (Fig. S6, B and C), suggesting that BipA proteins in the EPEC and K-12 strains play a differential role in flagella-mediated cell motility.

Interestingly, Krishnan and Flower (73) reported that the cold-sensitive phenotype of bipA deletion in K-12 was suppressed by the abolishment of RluC, which functions as a pseudouridylation-modifying enzyme at three sites in the 23S rRNA near the peptidyltransferase center (74, 75). This finding supports our results because the function of BipA is closely related either to the structure or the function of the ribosome. Notably, the results showed that bipA deletion decreased the expression of cps-lacZ, a capsule synthesis gene fused to lacZ, and double deletion of rulC and bipA enhanced expression of cps-lacZ. This inconsistent result must be further investigated.

Apparently, tyrosine phosphorylation of BipA in EPEC is important for its virulence and GTPase activity (76, 77). Although the primary sequences of BipA in EPEC and E. coli K-12 are completely identical, BipA in E. coli K-12 did not undergo phosphorylation at tyrosine residues, implying the presence of an additional phosphorylation factor in EPEC. This tyrosine phosphorylation is known as a rare reaction in bacteria (78). Nevertheless, a recent report demonstrates that 117 proteins, including translation-associated proteins and chaperones, are phosphorylated on tyrosine in E. coli K-12 (79). Thus, to examine whether the essential function of BipA at low temperature depends on phosphorylation, three conserved tyrosine residues, Tyr-164, -472, and -519, were mutated to phenylalanine individually or in combination in pACYC184BipA (Fig. S7A). As shown in Fig. S7B, ESC19 cells expressing BipA mutants exhibited similar growth to ESC19 harboring pACYC184BipA, suggesting that the substitution of conserved tyrosine residues with phenylalanine in BipA has no effect on its function at low temperature. Furthermore, Western blot analysis using anti-phosphotyrosine antibodies did not detect BipA proteins (Fig. S7C), implying that BipA may not be phosphorylated at 37 or 20 °C. Collectively, it seems that E. coli K-12 BipA is not phosphorylated under the tested condition; however, we cannot rule out the possibility that phosphorylation and dephosphorylation is too transient to be detected. Many proteins were tyrosine-phosphorylated (Fig. S7C), consistent with the findings of Hansen et al. (79).

Our results demonstrate that BipA is a novel ribosome-associated translational GTPase that is induced by CRP at low temperature, is involved in the assembly of L6 ribosomal protein and/or presumably in ribosome stability, and possesses chaperone activity. Furthermore, given that bipA deletion (ESC19) causes pleiotropic phenotypes, such as capsule production, motility, ribosome biogenesis, and cold sensitivity, it is very much likely that BipA is a global regulator through ribosome association or ribosome-mediated translational regulation.

### Materials and methods

**Bacterial strains and growth conditions**

*E. coli* strains were grown in LB medium with chloramphenicol (50 μg/ml), kanamycin (50 μg/ml), or ampicillin (100 μg/ml) as needed. The *E. coli* strains used in this study are listed in Table 1. To construct MG1655 *bipA::kan (ESC19)* and

| Strains | Description | Source or reference |
|---------|-------------|---------------------|
| MG1655 | F′ λ (λrp1-ci-t2) F’avr-6b(rph-1) | Ref. 5 |
| ESC19  | bipA::kan, MG1655 | This study |
| ESC25  | crp::kan, MG1655 | This study |
| JW5751 | bipA::kan, BW25113 | Ref. 80 |
| JW5702 | crp::kan, BW25113 | Ref. 80 |
| BL21(DE3) | F′ompT lacB57 (T_n-m_n) gal dcm (DE3) | Novagen |
This study

primer sets presented in the template and the primer sets listed in Table 2.

**Table 2**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| Cloning     |                  |                     |
| bipA-100bp-U| CTCCTGCAATACCTGTG  | This study          |
| bipA-100bp-D| CCAAGAGCTTAAAGGGAG | This study          |
| dnaK-3-HindIII| CAAGCTTTTATTATTGTCTT | This study          |
| dnaK-5-Ndel | CGCAATTGAGCGTAATGTCG | This study          |

**Construction of BipA truncation mutant**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-3-HindIII | AGAACTTTTTAATGCTTTTCGAGT | This study          |
| BipA-303–3-HindIII | ATAGAGCTTTTTATTCAAGAGGATGTTGG | This study          |
| BipA-390–3-HindIII | GGGAAGATTTCGAGAATCGTTCGGAGT | This study          |
| BipA-480–3-HindIII | ATAGAGCTTTTTATTCAAGAGGATGTTGG | This study          |
| BipA-5-Ndel | GCAGATGGTTGAGCGTTCG | This study          |
| BipA-49–5-Ndel | GAAGATTCGAGGAGGAGGAAGCTGGA | This study          |
| BipA-149–5-Ndel | ATACGGAGTTTAATGCTCAAGGA | This study          |

**Construction of BipA mutant**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| bipA-N128D-F | AAGCTTTTTTATGGGGAAGGGGTAAGTGAGTGGTGTTTCAAGAGGATGTTGG | This study          |
| bipA-N128D-R | CGGTACCTTTTTTTTATTCAAGAGGATGTTGG | This study          |
| bipA-Y164F-F | GCAGATGGTTGAGCGTTCG | This study          |
| bipA-Y164F-R | GCAGATGGTTGAGCGTTCG | This study          |
| bipA-Y472F-F | GCAGATGGTTGAGCGTTCG | This study          |
| bipA-Y472F-R | GCAGATGGTTGAGCGTTCG | This study          |
| bipA-Y519F-F | GCAGATGGTTGAGCGTTCG | This study          |
| bipA-Y519F-R | GCAGATGGTTGAGCGTTCG | This study          |

**qRT-PCR**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| bipA-RT-3   | GAACGTTGTTCAAAACCGCC | This study          |
| bipA-RT-5   | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |
| cspA-RT-3   | CAAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| cspA-RT-5   | CAAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| rrsA-RT-3   | GAGAGGTTGTTCAAAACCGCC | This study          |
| rrsA-RT-5   | GAGAGGTTGTTCAAAACCGCC | This study          |

**EMSA**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BCBS-I-F    | AGAGCTTTTTTATGGGGAAGGGGTAAGTGAGTGGTGTTTCAAGAGGATGTTGG | This study          |
| BCBS-I-R    | CAAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-F   | AGAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-R   | AGAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-III-F  | AGAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-III-R  | AGAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-mut1-F | AAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-mut1-R | AAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-mut2-F | AAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-mut2-R | AAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-mut3-F | AAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-mut3-R | AAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-mut4-F | AAGCTTTTTTATTCAAGAGGATGTTGG | This study          |
| BCBS-II-mut4-R | AAGCTTTTTTATTCAAGAGGATGTTGG | This study          |

**BipA-5-NdeI**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-100bp-U | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |
| BipA-100bp-D | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-149–5-NdeI**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-149–5-NdeI | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-15–5-NdeI**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-15–5-NdeI | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-303–3-HindIII**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-303–3-HindIII | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-390–3-HindIII**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-390–3-HindIII | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-480–3-HindIII**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-480–3-HindIII | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-5-Ndel**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-5-Ndel | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-49–5-Ndel**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-49–5-Ndel | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-149–5-Ndel**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-149–5-Ndel | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**BipA-15–5-Ndel**

| Primer name | Sequence (5’→3’) | Source or reference |
|-------------|------------------|---------------------|
| BipA-15–5-Ndel | GTGAGCTGGAGTGGTGTTTCAAGAGGATGTTGG | This study          |

**Plasmid construction**

DNA fragments of full-length and truncated bipA and dnaK were amplified by PCR of the MG1655 genomic DNA using the primer sets presented in Table 2. Each PCR fragment was subjected to blunt-end ligation into the Smal site of pUC19 (Table 3), and these subclones were digested with the appropriate restriction enzymes. Then each insert was ligated into pET28a, yielding pET28BipA and pET28BipA mutant proteins.

**Protein overexpression and purification**

Proteins used in this study were overexpressed in *E. coli* BL21(DE3). Cells transformed with pET-based vectors were cultured at 37 °C until an *A* <sub>600</sub> of 0.5–0.6 was obtained, after which isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. The cultures were then grown at 15 °C overnight (for His<sub>6</sub>-BipA and His<sub>6</sub>-BipA mutant proteins) or at 37 °C for 4 h (for His<sub>6</sub>-DnaK proteins). For the production of N-terminal His<sub>6</sub>-tagged GFP, BL21(DE3) cells were transformed with pEcoli-6xHN-GFPuv, and the induction conditions were the same as for His<sub>6</sub>-BipA. The induced cells were subsequently harvested by centrifugation at 4 °C, after which the cell pellets were washed with 10 mM Tris-HCl (pH 6.8) and subjected to a second centrifugation. To purify His<sub>6</sub>-BipA and His<sub>6</sub>-BipA mutants, the cell pellets were resuspended in 20 ml...
of Buffer A (20 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 2 mM β-mercaptoethanol (BME)), lysed by sonication, and centrifuged at 10,000 × g for 25 min at 4 °C to remove the insoluble fraction. The resulting supernatant was subjected to ultracentrifugation at 70,000 × g for 1 h at 4 °C in a Beckman 70Ti rotor to remove the membrane fraction. Next, the soluble fractions were applied to a Ni-NTA–agarose resin (Qiagen) column that had been equilibrated with Buffer A. The columns were washed with 40 column volumes of Buffer A, following which His6-tagged proteins were eluted with Buffer A containing 250 mM imidazole. The eluted proteins were dialyzed twice overnight against 2 liters of Buffer B without imidazole. For the purification of CRP-His6, BL21(DE3) cells transformed with pACYC184BipAY164F/Y472F/Y519F were cultured at 37 °C in LB medium with ampicillin until an A600 of 0.5–0.6 was obtained in 100 ml of LB medium. Polysomes were trapped by the addition of chloramphenicol to the culture at a final concentration of 100 μg/ml. Following an additional 3 min of incubation, the cells were harvested by centrifugation.

Sucrose density gradient sedimentation

Polysomes were prepared and resolved as described previously with minor modifications (82). In brief, strains MG1655 and ESC19 were grown at 20, 37, or 43 °C until an A600 of 0.5–0.6 was obtained in 100 ml of LB medium. Polysomes were trapped by the addition of chloramphenicol to the culture at a final concentration of 100 μg/ml. Following an additional 3 min of incubation, the cells were harvested by centrifugation for stringent response induction, SHX at a final concentration of 0.1 mM was added to cultures of MG1655 cells grown in LB medium until an A600 of 0.5–0.6 at 20 or 37 °C, followed by further incubation for 1 h at 20 °C or for 30 min at 37 °C, respectively. The cell pellet was then resuspended in 0.5 ml of Buffer BP (20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 100 mM NH4Cl, and 5 mM BME) and placed in a Beckman ultracentrifuge tube. Cells were then lysed by immersing the tube into a liquid nitrogen bath for 1 min, followed by thawing in cold water until no traces of ice remained. This freeze–thaw cycle was repeated twice, and the lysate was subsequently subjected to centrifugation at 100,000 × g for 10 min in a Beckman TLA100.4 rotor. Polysomes and subunits were resolved as described previously (83).

GTPase assay

Enzymatic assays were performed as described previously, with minor modifications (84). The release of free P1 was measured using the Malachite Green phosphate assay kit (BioAssay Systems), according to the manufacturer’s instructions. The reaction was conducted in a 100-μl reaction mixture of 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 20 mM MgCl2, 2 mM DTT, 128 μM GTP, and 0.2 μM protein for 20 min at 37 °C. The reaction was stopped by adding working reagent. The reaction mixture was incubated for 30 min at room temperature for color development. Optical absorbance was measured using a MultiskanTM GO microplate spectrophotometer (Thermo Scientific) at 620 nm.

### Table 3

| Plasmids | Description | Source or reference |
|----------|-------------|---------------------|
| pACYC177 | Ampβ, Kanα, ori p15A; cloning vector | New England Biolabs |
| pACYC184 | Cmβ, Tcα, ori p15A, cloning vector | New England Biolabs |
| pET28a  | His6-tagged expression vector | Novagen |
| pRelA   | relAβ, pACYC177 | Ref. 31 |
| pT7-crp_Ecoli | crpα, pT7-7 | This study |
| pBipA   | bipAβ, pACYC184 | This study |
| pACYC184BipA128D | bipA128D, pACYC184 | This study |
| pACYC184BipAY164F | bipAY164F, pACYC184 | This study |
| pACYC184BipAY472F | bipAY472F, pACYC184 | This study |
| pACYC184BipAY519F | bipAY519F, pACYC184 | This study |
| pACYC184BipAY164F/7472F | bipAY164F/7472F, pACYC184 | This study |
| pACYC184BipAY164F/7472F/519F | bipAY164F/7472F/519F, pACYC184 | This study |
| pT28DnaK | dnaKβ, pET28a | This study |
| pET28BipA | bipAβ, pET28a | This study |
| pET28BipA(1–303) | bipA1–303 aa, pET28a | This study |
| pET28BipA(1–390) | bipA1–390 aa, pET28a | This study |
| pET28BipA(1–480) | bipA1–480 aa, pET28a | This study |
| pET28BipA(49–607) | bipA49–607 aa, pET28a | This study |
| pET28BipA(149–607) | bipA149–607 aa, pET28a | This study |

aa, amino acids.
Chaperone-like activity of BipA

mg. Each value was averaged from at least three independent experiments.

qRT-PCR

MG1655 cells were grown in LB medium at 37°C for 3 h, and then cell culture was shifted to 20 or 43°C and incubated for 30 min. An equal number of cells were harvested by centrifugation based on the OD value. MG1655 cells harboring pACYC177 or pRelA were grown to an A600 = 0.6–0.7 at 37°C and then collected. For induction of the stringent response, MG1655 cells were grown in LB medium at 37°C for 3 h and shifted to 37 or 20°C. Then SHX was added to a final concentration of 0.1 mM, after which the cultures were incubated for an additional 30 min. Total RNAs were extracted using the hot phenol method with minor modifications (85). Pelleted cells were resuspended in 500 μl of solution A (20 mM sodium acetate, 10 mM EDTA, 0.5% SDS), and an equal volume of phenol was added, followed by incubation for 10 min at 60°C. After centrifugation for 10 min at 17,000 × g at 4°C, 400 μl of the aqueous phase was mixed with 40 μl of 3 M sodium acetate (pH 4.3). Next, 1 ml of ice-cold 100% ethanol was added to the mixture, followed by incubation for 30 min at −20°C and centrifugation for 10 min at 17,000 × g at 4°C. The pellet was washed with 100 μl of 100% ethanol and dissolved in 100 μl of distilled water. The RNA quality was assessed using agarose gel electrophoresis, and the RNA concentration was determined using a Thermo Scientific NanoDrop 2000. RNase-free DNase (Qiagen) was then used to remove genomic DNA contamination, according to the manufacturer’s instructions. After DNase treatment, DNase was removed with phenol, and RNAs were precipitated with ethanol. The quality and concentration of the total RNA were assessed as described above. Complementary DNA (cDNA) synthesis reactions were performed using the BioFact™ RT-Kit (MoIoney murine leukemia virus, RNase H−), RTase (BioFact), and 1 μg of total RNA. PCR was performed using 1 μl of cDNA and 2 pmol of each gene-specific primer (Table 2) in a 20-μl volume with 2× SYBR Green master mix (Qiagen). The reactions were carried out on a StepOne Plus real-time PCR system (Applied Biosystems) using the following cycling parameters: 95°C for 15 min, 40 cycles of denaturation at 95°C for 20 s, primer annealing at 55°C for 30 s, and extension at 72°C for 50 s. The 16S rRNA served as an endogenous reference, and relative expression levels were calculated using the comparative Ct method (86).

SDS-PAGE and Western blot analysis

Proteins were separated by SDS-PAGE using a 12.5% polyacrylamide gel, after which the proteins were transferred onto a PMV membrane (GE Healthcare). Anti-BipA antibodies were used as the primary antibody. For Western blot analysis, PageRuler prestained protein ladder (Thermo Fisher Scientific) was used.

EMSA

The BCBS I fragment was amplified by PCR, electrophoresed in agarose gel, and then purified. The WT and mutated BCBS II and BCBS III fragments were produced by annealing two single-stranded oligonucleotides with complementary sequences (Table 2). In the reaction mixture (10 μl), DNA fragments (20 nm) were incubated with increasing amounts of purified CRP-His6 at 37°C for 10 min in binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 200 μM cAMP). After the addition of 6× DNA loading dye (10 mM Tris-HCl (pH 7.5), 0.03% bromphenol blue, 0.03% xylene cyanol FF, 60% glycerol, and 60 mM EDTA), the reaction mixtures were loaded on 4% nondenaturing polyacrylamide gels in 0.5× TBE (Tris-borate-EDTA) buffer supplemented with 200 μM CAMP and electrophoresed at 100 V for 1 h at 4°C. Then gels were stained with the DNA SafeStain (Lamda Biotech) and visualized using ChemiDoc XRS+ (Bio-Rad).

Refolding assays

Acid denaturation and refolding of GFP were performed as described by Mares (87) with minor modifications. To generate 50 μM denatured GFP, 100 μM GFP solution was mixed with an equal volume of GFP-refolding buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2 mM BME, 20 mM MgCl2, 10% glycerol) containing 125 mM HCl and incubated at room temperature for 1 min. GFP refolding was started by diluting the 5 μl of denatured GFP in 495 μl of GFP-refolding buffer containing enzymes. Refolding of denatured GFP was monitored for 20 min at room temperature with excitation at 405 nm and emission at 535 nm using a Mithras LB 940 multimode microplate reader (Berthold). For denaturation and refolding of α-glucosidase, the assay was performed as described previously (48), in which 7.5 μM of α-glucosidase was denatured in 7.2 μl urea, 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, and 20 mM DTT at 25°C for 15 min. The denatured α-glucosidase was diluted 30-fold in α-glucosidase refolding buffer (40 mM HEPES-KOH (pH 7.5), 200 mM NaCl, 2 mM MgCl2) containing enzymes and incubated at 25°C. To measure α-glucosidase activity, 5 μl of the refolding reaction mixture was mixed with 20 μl of buffer supplemented with 60 mM potassium phosphate (pH 7.0), 0.1 mM GSH, and 0.9 mM p-nitrophenyl α-d-glucoside, followed by incubation at 37°C for 20 min. The chromogenic reaction was terminated by the addition of 100 μl of 0.1 M sodium carbonate, and the absorbance of each sample was determined at λ = 400 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific).

Statistical analysis

Results are presented as the mean ± S.D. of three independent experiments. The data were analyzed by unpaired two-way t test. The statistical analyses were made at significance levels as follows: NS, nonsignificant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

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