JOURNAL OF BACTERIOLOGY, Apr. 1993, p. 2321–2326
0021-9193/93/082321-06$02.00/0

Vol. 175, No. 8

AppppA-Binding Protein E89 Is the *Escherichia coli* Heat Shock Protein ClpB

EDWINA K. FUGE†* AND SPENCER B. FARR

Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, Massachusetts 02115

Received 11 June 1992/Accepted 12 February 1993

Dinucleotide AppppA (5′,5‴-P¹,P⁴-diadenosine tetraphosphate) is rapidly synthesized in *Escherichia coli* cells during heat shock. *apaH* mutants lack AppppN hydrolase activity and, therefore, contain constitutively high levels of AppppA, which affect several cellular processes. However, the precise role of AppppA remains undetermined. Photo-crosslinking experiments with radioactively labelled azido-AppppA have shown that a number of proteins, including heat shock proteins DnaK and GroEL, specifically bind to AppppA. Several other unidentified proteins (C40, C45, and E89) also bind strongly to AppppA. In this work, we have identified the AppppA-binding protein E89 as heat shock protein ClpB. In addition, since ClpB belongs to a family of proteins implicated in proteolysis, we have examined the effects of *apaH* mutants on protein degradation. Constitutively elevated levels of AppppA stimulate *lon*-independent proteolysis only in heat-shocked cells. We also show that overproduction of ClpB from a plasmid rescues *apaH* mutants from sensitivity to killing by heat.

AppppA (5′,5‴-P¹,P⁴-diadenosine tetraphosphate) is representative of a number of small polyphosphorylated dinucleotides synthesized in all living cells (5). The cellular concentrations of these nucleotides vary according to environmental factors such as temperature, oxidants, and viral infection (1, 3, 14, 19). On the basis of coincidental increases during heat shock, oxidative stress, and cell proliferation, AppppA has been postulated to be a molecular signal for induction of stress responses (2).

In *Escherichia coli*, there is a single hydrolase activity for AppppA, which is encoded by the *apaH* gene. On the basis of experiments using this gene, it has recently become clear that AppppA is not the molecular signal for the heat shock response in *E. coli*. *apaH* deletion mutants, which exhibit AppppA levels that are 100-fold higher than basal levels, do not constitutively express the heat shock or oxidative stress responses (8, 20). Furthermore, cells harboring a high-copy-number plasmid containing the *apaH* gene demonstrate normal heat shock protein synthesis, even though AppppA levels are severely reduced (25). Moreover, AppppA production lags behind heat shock protein synthesis (31).

Despite indications that AppppA is not the effector that signals thermal stress, several observations suggest that it nevertheless plays a substantial role in modulating the heat shock response. First, AppppA levels rise rapidly when the cell is exposed to heat (19). Second, *lysU*, which encodes lysyl tRNA synthetase, is itself a heat shock gene (5, 6, 31). Lysyl tRNA synthetase contributes significantly to AppppA production in *E. coli*. Third, *apaH* mutants exhibit increased sensitivity to killing by heat (14). Fourth, AppppA binds to heat shock proteins DnaK and GroEL as well as several unidentified heat shock proteins (E89, C40, and C45) (14).

Elevated AppppA levels cause other cellular defects in *E. coli*. *apaH* mutants (i) show diminished induction of catabolite-repressible genes (8, 20), (ii) are sensitive to killing by near-UV light, and (iii) are sensitive to killing by starvation; (iv) *apaH ksgA* mutants are defective in cell division (7a). We do not know how AppppA affects these processes, nor do we know whether it affects a few specific proteins or whether the effect is more general. Analysis of the mechanism of AppppA action has been hindered by the lack of clearly identified AppppA-binding proteins. In *E. coli*, the only evidence of a link between AppppA binding and a direct effect on function has been demonstrated with DnaK, in which AppppA alters in vitro the nucleotidase activity and the conformation of the DnaK protein (4, 7a, 30). However, a parallel effect in vivo has not been established.

In *E. coli*, several proteins of unknown function bind very strongly to AppppA (14). To begin studying the in vivo mechanism of AppppA action, we were interested in determining the identities of these proteins. In this report, we identify the strong AppppA-binding protein E89 as the ClpB protein. On the basis of this knowledge, we have examined the effects of elevated AppppA levels on proteolysis. We have also examined the ability of ClpB to rescue *apaH* mutants from thermosensitivity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in these experiments are *E. coli* K-12 derivatives. SF436 is an *apaH* mutant, which was derived from AB1157 by insertional inactivation of the *apaH* gene with a kanamycin resistance gene as described previously (8). SF894 (*lon*::Tn*10*) and SF887 (*lon*::Tn*10 apaH*::*kan*) were constructed by P1-mediated transduction of *lon*::Tn*10* into AB1157 and SF436, respectively, followed by selection for tetracycline resistance. The *clpB* (null) mutant and the plasmid-bearing pClpB strain were obtained from C. Squires and are described by Squires et al. (28). The *clpB* (null) mutation was created by insertion of a kanamycin resistance gene into *clpB*. pClpB carries the wild-type *clpB* gene as well as genes for ampicillin resistance and chloramphenicol resistance.

Preparation of protein extract. Cells were grown to mid-log phase in Luria-Bertani medium with the appropriate antibiotics (kanamycin, 50 μg/ml; ampicillin, 40 μg/ml; or chloramphenicol, 20 μg/ml). Cultures were chilled, pelleted by

* Corresponding author.
† Present address: Department of Biology, University of New Mexico, Albuquerque, NM 87131.

centrifugation, and resuspended in lysis buffer (50 mM MES [morpholineethanesulfonic acid]-KOH [pH 8.9], 10 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol). The cells were then lysed by freeze-thawing, and the cellular debris was cleared by centrifugation. Protein extract was dialyzed (with 50-kDa-cutoff dialysis tubing) overnight at 4°C in 25 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.6)–25 mM NaCl prior to separation by FPLC (fast protein liquid chromatography) ion-exchange chromatography.

For radioactive labelling of protein, cultures were incubated for 5 min with [^{35}S]Trans label (ICN) prior to pelleting and lysis.

FPLC ion-exchange chromatography of protein extract. Dialyzed protein extract (0.85 mg) was loaded onto a Pharmacia MonoQ HRS/5 anion-exchange column. Starting buffer was 25 mM PIPES (pH 6.6)–25 mM NaCl–10% glycerol. Extract was eluted in 25 mM PIPES (pH 6.6)–1 M NaCl–10% glycerol with a 30-ml linear gradient at a rate of 0.5 ml/min. Fractions (0.5 ml each) were collected, and the amount of protein was determined by the Bradford assay (Bio-Rad). Proteins in selected fractions were photo-crosslinked with [^{32}P]8-N_3AppppA and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Photoaffinity labelling and SDS-PAGE of protein extracts. Synthesis of [^{32}P]8-N_3AppppA and photo-crosslinking reactions were performed as described by Johnstone and Farr (14). Protein extract and 3 μl of [^{32}P]8-N_3AppppA (2.4 μM) were mixed (in a final volume of 40 μl) and spread as a thin film in the recessed top of a chilled Eppendorf tube. The above steps were performed under a 15-W red light in the dark. Samples were then UV irradiated at 265 nm at 3 W/m^2 for 5 min. For one-dimensional SDS-PAGE analysis, the photo-crosslinked samples were boiled for 5 min in 1 volume of 2× Laemmli buffer (10% glycerol, 5% β-mercaptoethanol, 10% SDS, 62 mM Tris [pH 6.8], 0.1% bromophenol blue). For two-dimensional (2D) SDS-PAGE, the proteins were added to 1 volume of buffer A (14) and then immediately stored at −20°C until use. Prior to photo-crosslinking, the salt concentration of FPLC-purified fractions was adjusted to 500 mM NaCl.

One-dimensional SDS-PAGE and 2D SDS-PAGE were performed as described by O'Farrell (23). After electrophoresis, the gels were silver stained (Stratagene kit), dried, and then exposed to Kodak X-Omat/AR film. The following ^{14}C-methylated proteins (obtained from Amersham) were used as molecular weight standards: myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (BSA) (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

Assay for degradation of abnormal proteins. Cells were grown at 30°C in supplemented M9 glucose medium lacking methionine. When the optical density of the culture at 600 nm was 0.5 to 0.6, puromycin (100 μg/ml) was added to the culture. After 10 min, [^{35}S]Trans label (10 μCi/ml) was added and half of the culture was shifted to 43°C for an additional 10 min. Cells were then washed, resuspended at the original cell density in growth medium containing unlabelled methionine (300 μg/ml), and returned to pretreatment growth conditions. At specified times, samples were added to trichloroacetic acid (10%) plus BSA (200 μg/ml) and placed on ice. The samples were then centrifuged, and radioactivity in the supernatant (trichloroacetic acid-soluble fraction) was determined by liquid scintillation counting. To estimate the total amount of incorporated radioactivity, a separate sample (at time zero) was evaluated directly. Protein degradation is expressed as the percentage of the total incorporated counts that is soluble in trichloroacetic acid as a function of time after removal of puromycin.

Heat sensitivity. Single colonies were inoculated into Luria-Bertani medium with the appropriate antibiotics and grown overnight at 30°C. In the morning, the cultures were diluted 1:100 into fresh medium containing ampicillin (40 μg/ml) and grown at 30°C. When the optical density of the culture at 600 nm was 0.25 to 0.35, half of the culture was shifted to 55°C, and at designated times thereafter, samples were diluted with 1× M9 salts, spread onto Luria-Bertani plates lacking antibiotics, and then incubated overnight at 30°C. Colonies were counted after a single overnight incubation.

RESULTS

AppppA binds to an 89-kDa heat shock protein. Several AppppA-binding proteins were previously identified (14). Two of these proteins, DnaK and GroEL, are known stress proteins. A third heat-inducible protein, E89, of unknown function, also bound AppppA to a greater extent than DnaK or GroEL (Fig. 1) (14).

To begin characterization of E89 and to eventually determine the gene encoding the protein, cell extract from *apaH*::*kan* mutants was dialyzed and fractionated by anion-exchange chromatography (see Materials and Methods). Individual fractions were photo-crosslinked with α-^{32}P-labelled azido-AppppA ([^{32}P]8-N_3AppppA) and then subjected to SDS-PAGE (14). An 89-kDa AppppA-binding protein was consistently eluted predominantly in a single fraction at approximately 300 mM NaCl (Fig. 2B, fraction 29). AppppA bound to the 89-kDa protein whether or not the culture had been heat shocked (Fig. 2B), although the amount of AppppA binding in fractions from heat-shocked cultures appeared to be greater. The difference may be due to a preponderance of the 89-kDa protein in heat-shocked cultures, visible in the silver stains of the gels (Fig. 2A).

The 89-kDa protein from fraction 29 was further examined by 2D PAGE (Fig. 3). Also, the photo-crosslinking pattern of fraction 29 and that of the nonfractionated sample from the same heat-shocked culture were compared. The prominent 89-kDa photo-crosslinking protein from fraction 29 (Fig. 3B) migrated to the same position as the protein previously identified as E89 (14) (Fig. 3A). In addition, a second, slightly smaller AppppA-binding protein also copurified with E89. The purification procedure did not appear to interfere with the AppppA-E89 (or smaller protein) interaction, because as much or more binding was observed for the fractionated sample as for the nonfractionated sample (Fig. 3). AppppA bound strongly to E89 even when the fraction was adjusted to 500 mM NaCl prior to photo-crosslinking (data not shown), thus supporting the existence of specificity in the binding interactions.

E89 is the product of the *clpB* gene. We considered the possibility that E89 might be ClpB because it was a heat shock protein and its position on 2D protein gels was near the location expected for ClpB (17, 28). We confirmed this suspicion when cell extract from a *clpB* deletion mutant was photo-crosslinked with [^{32}P]8-N_3AppppA; a protein corresponding to E89, which was apparent in the *clpB*$^+$ control extract (Fig. 4B), was not evident in the *clpB* deletion strain (Fig. 4C). In addition, a strain harboring a multicopy plasmid (pClpB) that encoded the ClpB protein showed greater E89-AppppA binding (Fig. 4D) than the *clpB*$^+$ strain, which

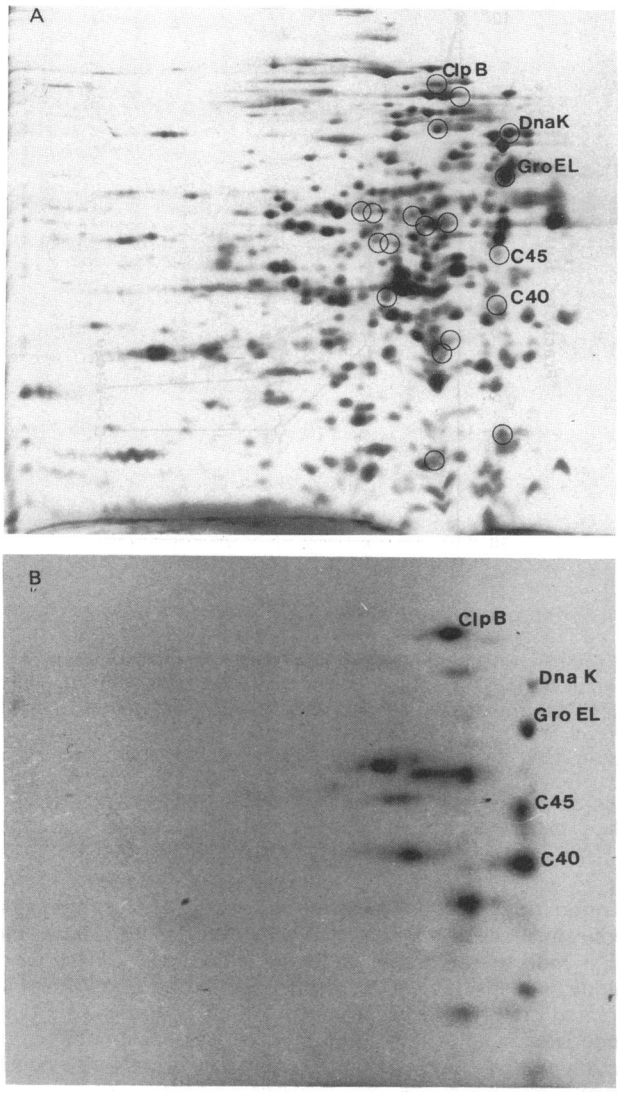

FIG. 1. 2D PAGE of [^{32}P]8-N_3AppppA photo-crosslinked *E. coli* protein extract. Extract was prepared individually from [^{35}S]L-methionine-labelled culture and spiked into the photo-crosslinked reaction mixture prior to 2D PAGE as described in reference 14. Two pieces of film were exposed to the gel simultaneously, and the positions of [^{32}P]8-N_3AppppA photo-crosslinked proteins that corresponded to [^{35}S]L-methionine-labelled proteins were identified by overlaying the two autoradiograms (14). (A) [^{35}S]L-methionine-labelled proteins from cells grown at 30°C; (B) [^{32}P]8-N_3AppppA photo-crosslinked proteins. Circled proteins in panel A are those proteins that are also photo-crosslinked by [^{32}P]8-N_3AppppA in panel B. The protein designated ClpB was previously identified as E89 (14). (Reprinted from an article by Johnstone and Farr [14] with permission of Oxford University Press.)

carried only a single copy of the *clpB* gene. Henceforth, we refer to E89 as ClpB.

A second heat shock protein, ClpB′, is also encoded by *clpB* (28). On 2D gels of extract from ^{35}S-labelled cultures, we observed a protein that could be ClpB′ on the basis of its molecular mass (68.5 kDa) and position relative to ClpB and DnaK (Fig. 4A). AppppA also bound to this protein, as shown in the corresponding autoradiogram of the identical sample (Fig. 4B), which had been photo-crosslinked with

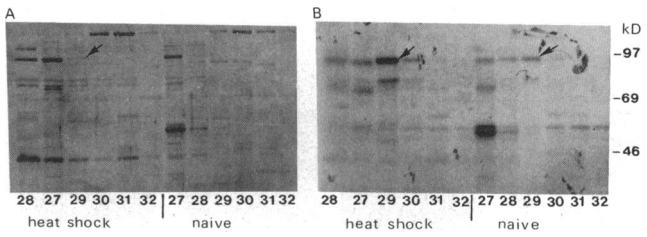

FIG. 2. Partial purification of E89 by ion-exchange chromatography. Protein extract was prepared from heat-shocked and non-heat-shocked (naive) cultures, dialyzed, and fractionated by FPLC ion-exchange chromatography (see Materials and Methods). Equal amounts of protein from designated fractions were photo-crosslinked with [^{32}P]8-N_3AppppA and separated by SDS-PAGE (see Materials and Methods). (A) Silver stain of the gel; (B) autoradiogram of the gel. Fractions are numbered at the bottom; molecular masses of protein standards are indicated on the right. Arrows, E89.

[^{32}P]8-N_3AppppA prior to gel electrophoresis. The increased representation of the photo-crosslinked protein in the pClpB extract (Fig. 4D) and its absence in the *clpB* mutant extract (Fig. 4C) suggest that the protein could be the previously identified ClpB′. Moreover, the protein appeared to copurify with ClpB during FPLC ion-exchange chromatography (Fig. 3).

Several other photo-crosslinked proteins appear to increase or decrease in parallel with the presence or absence of ClpB (Fig. 4C and D). Possibly, ClpB affects other AppppA-binding proteins, which coincidently have molecular weights and isoelectric points similar to those of ClpB. Further studies will be required to ascertain the significance of these additional differences.

Heat shock-stimulated proteolysis is enhanced in *apaH* mutants. We examined whether protein degradation was affected in *apaH* mutants. Since a significant portion of heat-inducible proteolysis is due to protease La (21), we were concerned that its presence could mask subtle effects that AppppA might exert on protein degradation. Therefore, we constructed a *lon apaH* mutant (see Materials and Methods) to examine degradation of both normal proteins and abnormal proteins. Abnormal proteins were generated by treating the culture with puromycin, which causes premature chain termination and, hence, truncated proteins. We were unable to consistently observe any statistically significant differences in basal-level protein degradation between the *lon apaH* mutants and the *lon apaH*$^+$ controls at any temperature (data not shown), nor was degradation of abnormal proteins in *lon apaH* mutants that were retained at

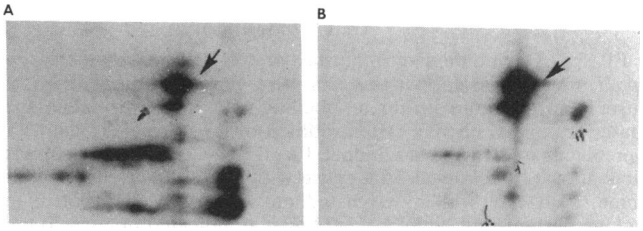

FIG. 3. 2D PAGE of the [^{32}P]8-N_3AppppA photo-crosslinked protein from dialyzed crude extract and FPLC fraction 29. Equal amounts of protein were photo-crosslinked with [^{32}P]8-N_3AppppA and then subjected to 2D PAGE as described in Materials and Methods. (A) Dialyzed extract; (B) dialyzed and FPLC-purified fraction 29. Arrows, E89.

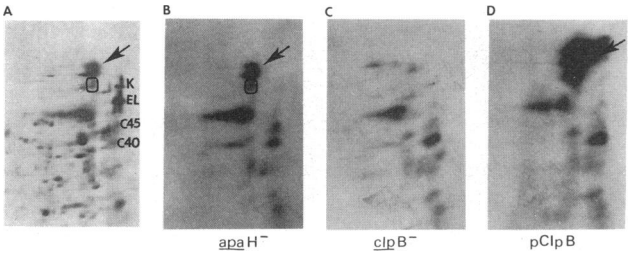

FIG. 4. [^{32}P]8-N_3AppppA photo-crosslinking reactions involving a *clpB* deletion strain and a strain carrying a multicopy plasmid, pClpB. Equal amounts of protein extract from heat-shocked cultures were photo-crosslinked with [^{32}P]8-N_3AppppA and separated by 2D PAGE. After electrophoresis, gels were exposed to X-ray film. Only a portion of the 2D gel is shown. (A) ^{35}S-labelled proteins from the *apaH* mutant; (B) [^{32}P]8-N_3AppppA photo-crosslinked proteins shown in panel A; (C) photo-crosslinked proteins from the *clpB*::*kan* mutant; (D) photo-crosslinked proteins from a pClpB plasmid-bearing strain. Arrows, ClpB protein. The possible ClpB′ protein is circled.

30°C significantly different from that in the *lon apaH*$^+$ controls. However, degradation of abnormal proteins in *lon apaH* mutants shifted to 43°C was 30 to 40% greater than that in *lon apaH*$^+$ controls that were incubated at either temperature (Fig. 5).

These results demonstrate that AppppA is involved in the degradation of abnormal proteins, although the biological consequences of this have not been ascertained. The results also indicate that constitutively high levels of AppppA, alone, are insufficient to affect degradation of puromycin-treated cultures, since proteolysis in the *lon apaH* mutants incubated at 30°C was not elevated. Some component(s) involved in the heat shock response is evidently also necessary.

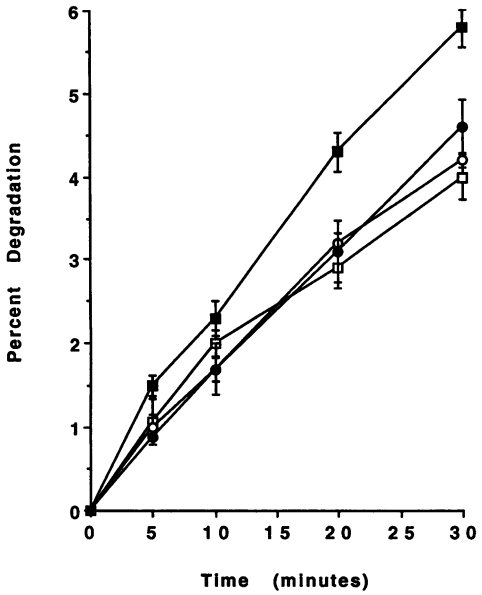

FIG. 5. Degradation of abnormal proteins in heat-shocked *lon apaH* mutants. Abnormal proteins were generated by addition of puromycin to cultures prior to labelling with [^{35}S]Trans label (see Materials and Methods). After addition of radiolabel, the cultures were incubated for an additional 10 min at 43 or 30°C, and this incubation was followed by a chase with excess unlabelled methionine. Cells were then harvested and resuspended in medium lacking puromycin. Samples were taken at indicated times, and the amounts of trichloroacetic acid-soluble radioactivity were determined (see Materials and Methods). The data are means and standard errors of the means for at least three independent experiments. ○ and ●, *lon*::Tn*10* mutants; □ and ■, *lon*::Tn*10 apaH*::*kan* mutants. Open and closed symbols represent 30 and 43°C treatments, respectively.

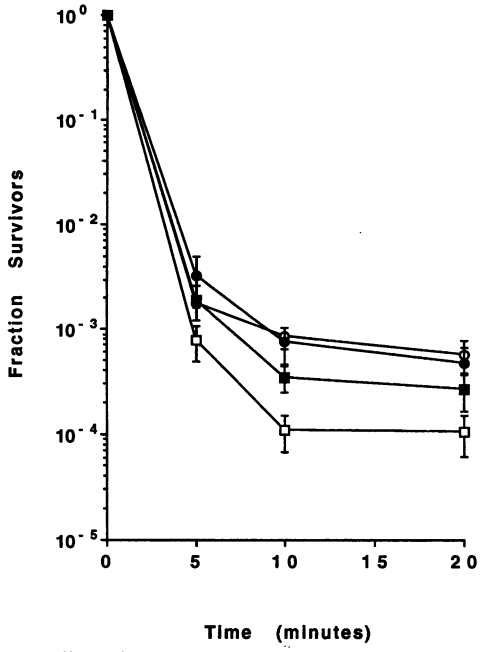

FIG. 6. Effect of pClpB on cell survival of *apaH* mutants at 55°C. Samples were taken at the indicated times after the shift from 30 to 55°C. The fraction of cells surviving was normalized to the number of viable cells at the time of the temperature shift. The data are means and standard errors of the means for four independent experiments. ○ and ●, wild type; □ and ■, *apaH* mutants. Open and closed symbols represent strains harboring pKK232, which is the parental vector plasmid, and pClpB, respectively.

Overproduction of ClpB rescues *apaH* mutants from heat sensitivity. *apaH* and *clpB* mutants are individually sensitive to killing by heat (14, 28). We reasoned that if high levels of AppppA were inhibiting ClpB and causing the sensitivity, then overproduction of ClpB might restore thermoresistance to *apaH* mutants. There was no statistically significant difference between survival of wild-type cells that harbored a multicopy plasmid (pClpB) carrying the *clpB* gene and survival of those carrying the control plasmid (pKK232) lacking the gene (Fig. 6). On the other hand, pClpB did enhance survival of the *apaH* mutant approximately three-fold over that of an *apaH* mutant harboring pKK232. The level of rescue was still only half that of the wild-type cells. These results suggest that at least part of the heat sensitivity of *apaH* mutants is due to an inhibitory effect of AppppA on ClpB activity.

DISCUSSION

Our results strengthen the assertion that elevated AppppA levels have physiological relevance during heat shock. We have identified the relatively strong AppppA-binding protein E89 as heat shock protein ClpB, thus bringing to three the number of characterized heat shock proteins that bind App-

ppA (the other two being DnaK and GroEL). In conjunction with heat shock, our data indicate that high levels of AppppA stimulate *lon*-independent degradation of abnormal proteins. We have also demonstrated that the thermosensitivity of *apaH* mutants is partially rescued by ClpB protein encoded by a multicopy plasmid.

ClpB belongs to a subclass of highly conserved proteins that include molecular chaperones, proteolysis regulators, and regulators of thermotolerance (29). A precise function has not been attributed to ClpB, but evidence suggesting that it may play an important role in protection or recovery from a variety of stresses is accumulating. In *E. coli*, *clpB* mutants exhibit a higher death rate at high temperatures (28; also, unpublished observations), underscoring the importance of ClpB in thermotolerance. In *Saccharomyces cerevisiae*, the ClpB homolog, Hsp104, is also important for recovery from thermal stress as well as a variety of other stressful conditions (26, 27). Interestingly, Hsp104 is generally not beneficial and may actually be detrimental for growth (27). This suggests that ClpB must necessarily be carefully regulated. In wild-type cells, AppppA synthesis lags behind heat shock protein synthesis (31); therefore, a role of AppppA may be to moderate the response through its interaction with specific proteins such as ClpB. A consequence of downregulation would be to return the cell to preinduction thermosensitivity. In *apaH* mutants, which are already sensitive to killing by heat at 55°C, the constitutively high level of AppppA may inhibit ClpB before it suitably performs its heat shock function. Overproduction of ClpB would increase the pool of active ClpB, thereby rescuing the cell from thermosensitivity, as we have observed.

Degradation of abnormal proteins may be an important function of the heat shock response (10). In *E. coli*, two energy-dependent heat-inducible proteases, La (7, 9) and Ti (12, 13, 15–18), have been characterized. La, a single-component protease consisting of the Lon protein, functions independently of Ti (16, 21). Protease Ti consists of a regulatory subunit, ClpA, and a proteolytic subunit, ClpP. Only the proteolytic subunit of Ti is induced by heat (18). On the basis of the 2D protein gel nomenclature of Pederson et al. (24), the designated positions for Lon and ClpP are H94.0 and F21.5, respectively (18, 22). We were unable to discern any significant binding of AppppA to either of these proteins (data not shown).

The amino acid sequence of ClpB is 50% identical to that of ClpA in the two regions that contain nucleotide binding sites (11). The reported molecular mass (80 kDa) and isoelectric point (7.0) for ClpA place this protein in the region of ClpB on 2D protein gels (13). A small spot, which could be ClpA, can be seen in the *clpB* mutant extract (Fig. 4C). Clearly, however, if this spot is ClpA, only a small amount of AppppA binding is apparent. Since ClpA is not induced by heat shock (15), other conditions may be necessary in order to discern any appreciable binding by AppppA.

Our results show that a *lon*-independent proteolytic system is enhanced by the *apaH* mutation. The modest stimulation of degradation suggests that a subclass of proteins or a specific protein is affected. Because of the conservation between ClpA and ClpB, it has been proposed that ClpB might be a heat-inducible regulatory subunit of Ti (11, 17). Possibly, AppppA affects an interaction between ClpP and ClpB and, thereby, proteolysis. However, there is presently no evidence demonstrating a direct involvement of ClpB in protein degradation (29). Alternatively, it has been proposed that ClpB may act as a chaperone and, as such, it could be involved indirectly in proteolysis (29). Further experiments will be required to determine whether AppppA is involved in protein degradation via its action on ClpB.

We have previously shown that AppppA also binds DnaK and GroEL, both of which have chaperone functions. Given the widespread occurrence of ClpB, DnaK, and GroEL homologs, it will be worthwhile to determine whether AppppA binds to cognate proteins in other cells as well. Initial experiments demonstrate binding to 70- and 90-kDa heat shock proteins in *Drosophila* Schneider cells and mammalian cells. It is, therefore, interesting to conjecture that a general function of AppppA may be to modify heat-inducible chaperoning activity.

ACKNOWLEDGMENTS

We thank C. Squires for the *clpB* deletion and pClpB strains and F. Goldberg for the *lon* deletion strain. We also thank X. Gidrol and D. Johnstone for many helpful discussions and F. Goldberg and M. Sherman for discussions on the proteolysis experiments.

This work was supported by an NIH IRSA 5F32ES05535 to E.K.F. and BRSG 72-081-8269-2 to S.B.F.

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