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Heat shock transcription factor δ^{32} is targeted for degradation via an ubiquitin-like protein ThiS in *Escherichia coli*

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ABSTRACT

The posttranslational modification of proteins with ubiquitin and ubiquitin-like proteins (UBLs) plays an important role in eukaryote biology, through which substrate proteins are targeted for degradation by the proteasome. Prokaryotes have been thought to degrade proteins by an ubiquitin independent pathway. Here, we show that ThiS, an ubiquitin-like protein, is covalently attached to δ^{32} and at least 27 other proteins, leading to their subsequent degradation by proteases, in a similar manner to the ubiquitin-proteasome system (UPS) in eukaryotes. Molecular biology and biochemical studies confirm that specific lysine in δ^{32} can be modified by ThiS. The results presented here establish a new model for δ^{32} degradation and show that *Escherichia coli* uses a small-protein modifier to control protein stability.

1. Introduction

The heat shock response (HSR) can be defined as the cellular response to increased temperature, during which a set of proteins termed heat shock proteins (HSPs) are up-regulated. These HSPs are usually regulated by the heat shock factor sigma 32 (δ^{32}) in *Escherichia coli* [1].

Following exposure to increased temperature, the intracellular level of δ^{32} transiently increases, leading to the transcription of heat shock related genes, with the ultimate aim of restoring the intracellular milieu to an environment appropriate for correct protein folding. Whilst the degradation process of δ^{32}, the global regulator in *E. coli*, has been unraveled, the mechanism how does δ^{32} recognize by protease remains elusive. To date, only a revised model for the activity and control of δ^{32} degradation has been reported [2]. The first step in this model involves the transport of δ^{32} to the inner membrane by the co-translational protein targeting machinery, composed of the SRP (Signal Recognition Particle) and the SRP receptor. Following localization in the inner membrane, δ^{32} is subject to chaperone-mediated activity control [3,4] and degradation, mediated by the essential protein FtsH [5]. Among a group of five purified ATP-dependent proteases (Lon, ClpAP, ClpXP, FtsH, and ClpYQ) [6], ClpYQ was also shown to directly degrade δ^{32} [7].

To improve our understanding of the recognition mechanism in the process of δ^{32} degradation, we investigated small-protein modifiers that can target substrate proteins for degradation. Two ubiquitin-like proteins (UBLs), ThiS and MoaD, have been identified in *E. coli* [8]. In organisms, modification by ubiquitin or UBLs targets substrates for degradation by the proteasome, a process which is crucial for proper cellular function [9]. However, relatively little is known about the *E. coli* ubiquitin-like proteins ThiS and MoaD. Here, we sought to investigate the function of ThiS and MoaD as small-protein modifiers. Biochemical studies have demonstrated that UBL proteins in eukaryotes, such as SUMO, modify a range of proteins involved in diverse cellular processes, including transcriptional regulators [10]. Recent studies of the UBL-like proteins Pup in *Mycobacterium tuberculosis* and Samp in *Haloferax volcanii* have elaborated the function of these UBLs in prokaryotes [11,12]. Pup and Samp can been specifically conjugated to proteasome substrates. Furthermore, in the yeast *Saccharomyces cerevisiae* the cell type-specific transcriptional repressor MATα2 has been reported to be ubiquitinated [13]. Taken together, these results suggest a rationale to further investigate the of δ^{32}, with a specific view to understanding the actions of ThiS and MoaD on δ^{32}.

To gain further insights into δ^{32} modification in *E. coli*, we purified His tagged δ^{32} and analyzed the relationship between δ^{32} and ThiS or MoaD by mass spectrometry. We observed that the heat

Abbreviations: IPTG, isopropyl-β-d-thiogalactoside; UPS, ubiquitin-proteasome system; UBLs, ubiquitin-like proteins; HSR, heat shock response; CBB, coomassie Brilliant Blue; Co-IP, Co-Immunoprecipitation..

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shock transcription factor δ^{32} was modified *in vivo* and *in vitro*. Surprisingly, we discovered that the signature tag of ThiS was covalently linked to δ^{32} and we also identified 27 ThiS conjugates. These results imply that δ^{32} is likely to be degraded by an ubiquitin-like modification pathway and the control of protein stability by ubiquitin-like proteins, such as ThiS, does occur in *E. coli*.

2. Materials and methods

2.1. Strains, plasmids, primers and culture conditions

Strains, plasmids and primer sequences are listed in Supplementary Table S4. *E. coli* strains were grown in Luria–Bertani broth or on Luria–Bertani-agar plates (Difco, NJ, USA). Antibiotic concentrations were as follows for *E. coli*: 100 μg/ml kanamycin and ampicillin, 25 μg/ml for chloramphenicol. Isopropyl-b-D-thio-galactopyranoside was used at 0.5 mM.

rpoH, thiS, moaD and *clpX* were sequentially cloned into pET28a(+). In order to construct plasmid for co-purification of δ^{32}~ThiS complex, *rpoH* was cloned into multiple cloning sites-1 of pETDuet-1 digested with *BamH*I and *Sal*I, then *this* was cloned into multiple cloning sites-2 with *Nde*I and *Xho*I digesting. To construct plasmid for bacterial two hybrid system, *thiS* and *moaD* were cloned into the *BamH*I and *Xho*I sites of pTRG; *clpA*, *clpE*, *clpP*, *clpX*, *clpY*, *hslU* an *ftsH* were cloned into pBT using *Not*I and *Xho*I.

For analysis of *E. coli* lysates, 10 ml cultures were grown to an optical density at 600 nm of approximately 0.4–0.6. Volume of 1 ml bacteria was collected at indicted time point after IPTG added. Centrifuged at top speed for 30 s at 4 °C, cells were boiled in 50 μl of 2xSDS-PAGE loading buffer.

2.2. SDS-PAGE and western blotting analysis

For western blotting analysis, proteins were separated on 12% SDS–polyacrylamide gel electrophoresis (PAGE) gels. Proteins were transferred onto 0.22 μm Polyvinylidene Fluoride using a wet transfer system (Bio-Rad, CA, USA), and incubated with antibodies to δ^{32}, Flag-His_6-ThiS, Flag-His_6-MoaD or His_6-clpX. Horseradish peroxidase-coupled rabbit secondary antibodies were used according to the manufacturer's instructions (Santa cruz, TX, USA). Horseradish peroxidase was detected using Immobilon Western Chemiluminescent HRP Substrate (Millpore, MA, USA).

2.3. The bacterial two-hybrid system

For confirmation the interaction between thiS, moaD and clpX , the bacterioMatch® II Two-Hybrid System Vector Kit (Stratagene, CA, USA) was used. Fifty ng pBT bait vector plus pTRG target vector was then co-transformed to report strain, plated 20 μl aliquots of the cell suspension on nonselective screening medium (no 3-AT) and 200 μl on selective screening medium (5 mM 3-AT), respectively. Incubating the plates at 37 °C for 24 h, the apparent colonies were grown in $M9^+$ His-dropout broth with Tetracycline/Chloramphenicol at 37 °C overnight. To verify the interaction between the bait and target proteins, the cultures of putative positive colonies were streaked on dual selective screening medium (5 mM 3-AT + 12.5 μg/ml streptomycin).

2.4. Modification in E. coli

To examine the modification of proteins in *E. coli*, strains were incubated at 37 °C with shaking until an optical density of 0.6 at 600 nm was reached. Cultures were then induced with 0.5 mM IPTG and incubated for two hours. A volume of 1 ml of the induced cultures was collected and bacteria were isolated by centrifugation for 1 min at top speed in microfuge tubes at 4 °C. Bacteria were resuspended in 30 μl sterile water, mixed with 5xsample buffer and boiled for 10 min before analysis by SDS–PAGE and western blotting.

2.5. Proteomic analysis

Purified proteins were visualized on a 12% SDS–PAGE gel and excised from the gel for MS/MS analysis (Done in Center of Biomedical Analysis, Tsinghua University, BJ, China). The gel slice was divided into 4 bands, each of which was cut into cubes of approximately 2 mm and transferred into 1.5 ml Eppendorf tubes.

3. Results and discussion

3.1. Modification of δ^{32} in vitro and in vivo

Proteins can be modified by Pup in *E. coli* in a reconstituted system [14], but the pupylation of transcription factors has not yet been reported. We noticed that δ^{32} could be modified in *E. coli*, which suggested that an undiscovered degradation pathway for δ^{32} in *E. coli*. To begin with, we expressed δ^{32} in *E. coli* from the vector pET28a using the IPTG inducible T7 promoter system. After the addition of IPTG, we used western blotting to detect δ^{32} at indicted time points with an anti-δ^{32} monoclonal antibody (Neoclone). Two μg whole protein was loaded for western blotting analysis. As expected, modified δ^{32} was observed as distinct bands after 10 min (Fig. 1A upper panel). And at 0 min (without IPTG), modified δ^{32} was observed as well, which may be because of the basal expression. Accordingly, the amount of HSPs was slightly increased after the induction of δ^{32} expression from the T7 promoter (Fig. 1A lower panels). We subsequently purified δ^{32} from *E. coli*, followed by western blotting analysis, where we were able to detect higher-molecular-weight δ^{32} as well (Fig. 1B). Additionally, previous study showed that δ^{32} might be phosphorylated because two forms of δ^{32} were observed in the isoelectric dimension of two-dimensional gels, but phosphorylation of δ^{32} was not able to be detected [15,16]. To confirm that the higher-molecular-weight proteins were related to δ^{32}, purified δ^{32} was separated by SDS-PAGE and stained with CBB (Fig. 1C). The bands corresponding to the different forms of δ^{32} were excised from the gels, trypsin digested and identified by mass spectrometry (MS). Using this approach, we confirmed that all of the higher-molecular-weight proteins were related to δ^{32}. Surprisingly, the ubiquitin-like proteins ThiS and MoaD [17], and homologues of enzymes associated with sulfur-activation, such as MoaB, ThiF and ThiE, were also identified (Supplementary Table S1). ThiS and MoaD, which are closely related to the eukaryotic URM1, sulfur in the form of a thiocarboxylate on the terminal glycine, just as the thioester linkages of UB/UBLs are formed during the conjugation process [8]. Furthermore, both ThiS and MoaD are adenylated by the enzymes ThiF and MoeB, respectively, prior to the acceptance of sulfur from the donor cysteine [18]. ThiF and MoeB are related to the Ub-conjugating E1 enzymes, which exhibit a characteristic architecture [19]. Thus, we speculated that δ^{32} modified by ThiS or MoaD.

Our MS analysis also detected the proteins GshA and BirA. GshA belongs to the PafA family of proteins [11] that are related to the γ-glutamyl-cysteine synthetases and glutamine synthetases [20], whilst BirA is a Group II biotin protein ligase [21]. The activities of these identified enzymes suggests that they responsible for conjugating activated ThiS or MoaD to δ^{32}. The potential ThiS or MoaD modification sites of δ^{32} were analyzed by tandem mass spectrometry (MS/MS) using established methods [22]. We identified one lysine site in δ^{32} that appeared to be attached to the ThiS peptide fragment N-EQWAQHIVQDGDQILLFQVIAGG-C, although

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