Evidence for an active role of the DnaK chaperone system in the degradation of σ^{32}

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Abstract Under non-stressed conditions in *Escherichia coli*, the heat shock transcription factor σ^{32} is rapidly degraded by the AAA protease FtsH. The DnaK chaperone system is also required for the rapid turnover of σ^{32} in the cell. It has been hypothesized that the DnaK chaperone system facilitates the degradation of σ^{32} by sequestering it from RNA polymerase core. This hypothesis predicts that mutant σ^{32} proteins, which are deficient in binding to RNA polymerase core, will be degraded independently of the DnaK chaperone system. We examined the in vivo stability of such mutant σ^{32} proteins. Results indicated that the mutant σ^{32} proteins as similar as authentic σ^{32} were stabilized in Δ*dnaK* and Δ*dnaJ*/Δ*cbpA* cells. The interaction between σ^{32} and DnaK/DnaJ/GrpE was not affected by these mutations. These results strongly suggest that the degradation of σ^{32} requires an unidentified active role of the DnaK chaperone system. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Protein turnover; FtsH; DnaK; Heat shock response; RNA polymerase

1. Introduction

Regulated proteolysis is an important fundamental process in the cell. Detrimental or useless proteins are selectively degraded by proteases. Especially, a set of proteins which are required for a limited period or under particular conditions, for example, regulatory proteins of the cell cycle or of the stress-mediated response, are removed rapidly when they are not required [1]. σ^{32}, a regulatory protein of the 'classical' heat shock response in *Escherichia coli*, is a well-known example of such unstable regulatory proteins [2,3]. Under non-stressed conditions, σ^{32} is very unstable (half-life < 1 min) but stabilized transiently after heat shock [4].

FtsH (also referred to as HflB), a membrane-associated, ATP-dependent protease, degrades σ^{32} in vivo and in vitro, and has a central role in the proteolytic turnover of σ^{32} [5–7]. FtsH is an essential protein and is a member of the AAA family [8,9]. FtsH degrades both membrane and cytoplasmic proteins other than σ^{32}, such as uncomplexed SecY [10], F$_o$ α subunit of proton ATPase [11], YccA protein [12], LpxC [13], λ CII [14], CIII [15], and Xis proteins [16], and SsrA-tagged

proteins [17]. FtsH forms a multimer and makes a complex with a pair of membrane proteins, HflK and HflC [18].

It has been believed that the DnaK, DnaJ and GrpE chaperone system plays an important role in degradation of σ^{32}, since σ^{32} is stabilized under conditions where the activity of DnaK chaperone system is reduced, e.g. in *dnaK*, *dnaJ* and *grpE* mutants [19], when the cellular level of DnaK/DnaJ is reduced [20], or when abnormal proteins, substrates of the DnaK chaperone system, are overproduced in the cell [21]. The DnaK chaperone system is one of the most abundant chaperone systems and plays a role in preventing aggregation and assisting refolding of protein [22]. Accordingly, the DnaK chaperone system senses the cellular level of misfolded or abnormal proteins, then controls the stability and the activity of σ^{32} [2,23]. However, the reason why the DnaK chaperone system is required for the rapid turnover of σ^{32} in vivo is largely unknown.

It has been proposed that the sequestration of σ^{32} from RNA polymerase core (RNAPcore) is required for the rapid turnover of σ^{32} and DnaK and DnaJ compete with RNAP for binding to σ^{32} then keep σ^{32} in a state sensitive to proteolysis [20,24]. If this hypothesis is correct, mutant σ^{32} proteins, which are deficient in RNAPcore-binding, will be degraded independently of the DnaK chaperone system. We examined the stability of such mutant σ^{32} proteins [25,26]. Results indicated that the mutant σ^{32} proteins still require the DnaK chaperone system for their rapid degradation.

2. Materials and methods

2.1. Strains, plasmids and media

Derivatives of *E. coli* strain W3110 (F$^-$ IN(*rrnD-rrnE*)*1*), AR3307 (W3110 *zad220*::Tn*10*), AR3291 (W3110 *zad220*::Tn*10 sfhC21 ΔftsH3*::*kan*), AR7071 (W3110 Δ*dnaK52*::*cat*), AR7051 (W3110 *zad220*::Tn*10 sfhC21 ΔftsH3*::*kan ΔdnaK52*::*cat*) and AR7222 (W3110 *cbpA*::*kan dnaJ*::Tn*10-42*), were described [7]. AR5088 (BL21[DE3] *zad220*::Tn*10 sfhC21 ΔftsH3*::*kan*) was also described [27].

Plasmids carrying *rpoH* alleles in a pBR322-derived vector were described (phis series; [25,26]). The *Eco*RI–*Hin*dIII fragments of these plasmids, which contained the *rpoH*-his gene, were subcloned into the multi-cloning site of a pSC101-derived vector pFN476 [28]. Resulting plasmids are pAR7011 (wild-type σ^{32}-c-his), pAR7012 (Q80R-c-his), pAR7013 (Q80N-c-his), pAR7016 (F136L-c-his), pAR7017 (wild-type σ^{32}-n-his), pAR7018 (L278W-n-his). pACYCQ was constructed by subcloning of a *Sal*I fragment of pDMI,1 [29] containing the *lacI*^{q} gene into the *Sal*I site of pACYC184. Plasmid pSTD430 is a derivative of pUC119 carrying the *zip-ftsHΔTM-myc* under the *lac* promoter [30].

L and M9 media were used. Antibiotics were added at the following concentrations when needed: ampicillin (50 μg/ml); tetracycline (12 μg/ml); chloramphenicol (20 μg/ml); kanamycin (25 μg/ml). Isopropyl

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β-D-thiogalactopyranoside (IPTG) was added to the medium for induction of the genes under the *lac* promoter.

2.2. Determination of the stability of σ³²

The procedures were described previously [7]. The radioactivity incorporated into σ³² was quantified by BAS2000A photo imager (Fuji Film Co.).

2.3. SDS–PAGE and Western blotting

Gel electrophoresis was carried out according to Laemmli [31] using 10 or 12% SDS–polyacrylamide gels. Gels were stained by Coomassie brilliant blue (CBB) or developed by Western blotting. Western blotting was carried out as described [32], except that immunoblots were developed by Renaissance Western blot chemiluminescence reagent Plus (NEN) according to the manufacturer's instructions. Membranes were exposed to X-ray films (RX; Fuji Film Co.). Stained gels or developed films were scanned by a flat-head scanner.

2.4. Purification of his-tagged σ³² by immobilized metal ion affinity chromatography (IMAC)

The experiments were performed according to Gemar et al. [29] with some modifications. Cells expressing σ³²-his from plasmid were grown in 200 ml of L medium containing 0.25% glucose at 30°C to a cell density corresponding to 60 Klett units. After the addition of IPTG (final concentration 1 mM), the cultures were further incubated for 2 h and cells were harvested by centrifugation. Cell pellets were suspended with buffer X (50 mM potassium phosphate (pH 7.9), 300 mM KCl, 50 mM Ile, 50 mM Phe) containing 0.1 mg/ml of lysozyme and 1 mM PMSF and subjected to sonication. Cell lysates were centrifuged for 30 min at $35\,000 \times g$, the supernatant was loaded onto a Ni^{2+}-conjugated Hi-trap Chelating column (1 ml; Pharmacia) at a rate of 1.0 ml/min. The column was subsequently washed with 10 ml of buffer X then with 5 ml buffer X+15 mM imidazole. Ni^{2+}-bound proteins were eluted with 12 ml of 15–200 mM linear gradient of imidazole in buffer X, and 0.5 ml of fractions was collected.

3. Results

3.1. RNAPcore-binding ability of σ³² does not affect its stability in vivo

We examined the stability of representative mutant σ³² proteins, which are deficient in core-binding ([25,26], Fig. 1). Alleles of *rpoH*, *rpoH173*, *rpoH185* and *rpoH112* were initially isolated as suppressors of *rpoD285*, which encodes a temperature-sensitive house-keeping σ factor, σ⁷⁰ [33], and these mutant σ³² proteins, Q80R, Q80N, F136L and L278W, do not compete with σ⁷⁰ in binding to RNAPcore in vitro [25,26]. His-tagged, these mutant σ³² proteins were expressed from a low-copy plasmid and their stability was measured in wild-type, ΔdnaK, ΔftsH and ΔdnaJ/ΔcbpA cells. Under the condi-
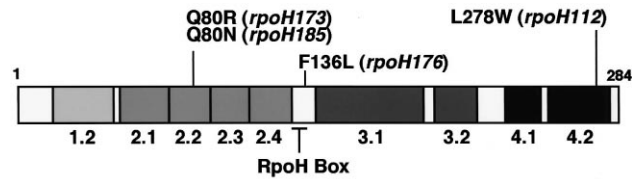

Fig. 1. Schematic representation of the mutations of σ³² mutants used in this study. The allele name and the amino acid substitution of each mutant are shown. Shaded boxes represent conserved regions (1.2–4.2) among σ factors according to Lonetto et al. [34]. The RpoH box is a conserved region among RpoH homologues [35].

tions used, where the expression of σ³²-his was not induced, the level of these mutant σ³²-his proteins expressed from the plasmid was ∼3-fold of that of authentic σ³² expressed from the *rpoH* gene on the chromosome of the host cell (Fig. 2). Both overexpression of σ³² and deletion of the *rpoH* gene alter the cellular levels of heat shock proteins including the components of the DnaK system and ATP-dependent proteases, which significantly affect the stability of σ³². His-tagged σ³² can be separated from authentic σ³² by SDS–PAGE (Fig. 2).

Results are shown in Table 1. Wild-type σ³² with a his-tag as well as authentic σ³² showed clear dependency in their degradation to the DnaK chaperone system and FtsH protease, although it was slightly unstable than authentic σ³². Thus, the his-tagging and expression from a plasmid do not significantly affect the stability of σ³². Three core-binding mutants (Q80R, Q80N and F136L) were very unstable (half-life < 1 min) in wild-type cells but highly stable (half-life > 100 min) in ΔftsH cells, indicating that their degradation is FtsH-dependent as is wild-type σ³². In contrast to the hypothesis that core-binding mutants are degraded independently of the DnaK chaperone system, these mutants were stabilized by the ΔdnaK or ΔdnaJ/ΔcbpA mutation as was wild-type σ³². These results clearly indicate that the DnaK chaperone system is required for the rapid turnover of core-binding mutant σ³² proteins, too. Mutant L278W was much unstable in ΔdnaK and ΔcbpA/dnaJ cells. However, it was also significantly unstable in ΔftsH cells. The L278W mutation may alter the conformation of σ³², so that it becomes hypersensitive to degradation by other proteases.

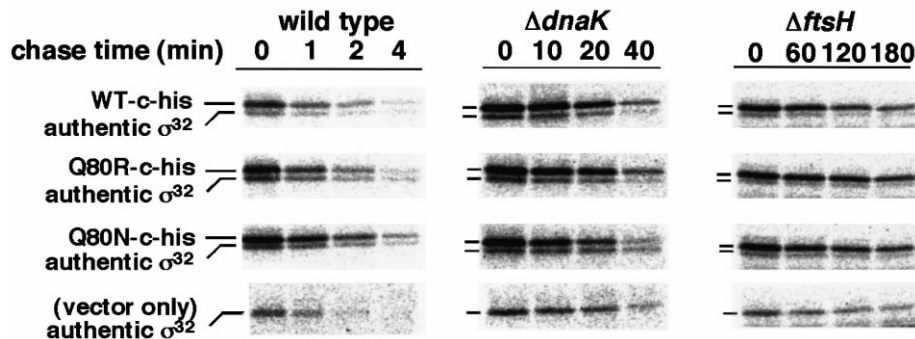Fig. 2. Stability of his-tagged mutant σ³² proteins. AR3307 (WT), AR7071 (ΔdnaK), AR7222 (ΔdnaJ/ΔcbpA) and AR3291 (ΔftsH sfhC) cells carrying pAR7011 (wild-type), pAR7012 (Q80R), pAR7013 (Q80N) or pFN476 (vector) were pulse-labeled for 1 min at 30°C and chased with unlabeled methionine and cysteine for 1 min (AR3307), 3 min (AR7071, AR7222) or 20 min (AR3291) before taking samples. Samples were taken at the indicated time and subjected to immunoprecipitation and SDS–PAGE.
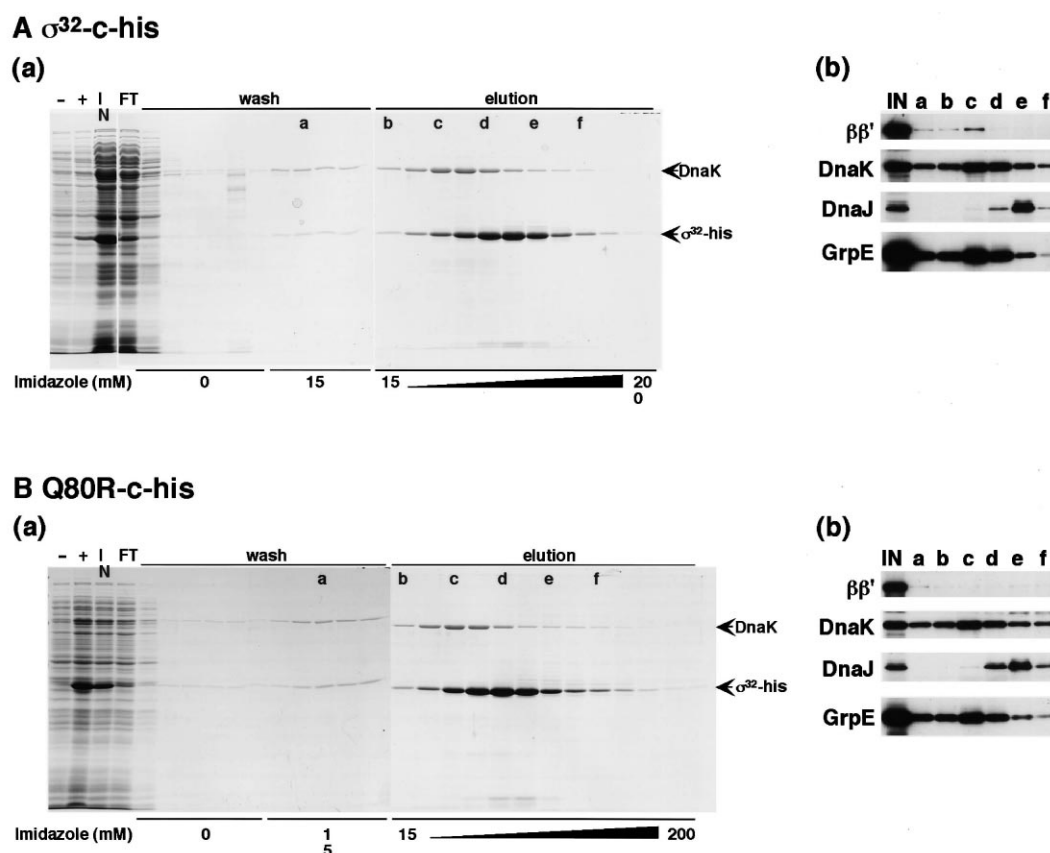
Fig. 3. Co-purification of members of the DnaK chaperone system and ββ′ subunits of RNA polymerase with his-tagged σ^{32} by IMAC. AR5088 (BL21[DE3] *sfhC ΔftsH*) cells carrying pACYCQ (pACYC184 *lacI*[q]) and pAR7011 (pFN476 T7p-*rpoH*-c-his; A) or pAR7012 (pFN476 T7p-*rpoH(Q80R)*-c-his; B) were grown in L medium containing 0.25% glucose at 30°C. After the addition of IPTG (final concentration 1 mM), the cultures were further incubated for 2 h and cells were harvested by centrifugation. Cells were subjected to IMAC analysis as described in Section 2. Samples of the cell culture taken before (−) and after (+) IPTG induction, of the lysate loaded to the column (IN), of the flow through fraction (FT) and of the fractions during wash and elution phases were analyzed by SDS–PAGE and visualized by CBB staining (a). Samples of IN and several fractions (a–f; indicated on [a]) were analyzed by Western blotting with anti-RNAPcore, anti-DnaK, anti-DnaJ or anti-GrpE antiserum (b).

3.2. The interaction between σ^{32} and the DnaK chaperone system is not affected by the core-binding deficient mutations

These mutations may affect the interaction of σ^{32} with members of the DnaK chaperone system. Binding of DnaK, DnaJ and GrpE with his-tagged σ^{32} was examined by IMAC (Fig. 3). DnaK, DnaJ and GrpE were co-eluted with Q80R-c-his in the same profile that they were co-eluted with wild-type σ^{32} (Fig. 3). In contrast, ββ′ subunits of RNAPcore were co-eluted with wild-type but not with Q80R. Q80N and F136L

mutants showed essentially the same elution profiles as that of Q80R (data not shown).

3.3. A soluble variant of FtsH requires the DnaK chaperone system in the degradation of σ^{32} in vivo

Another possible role of the DnaK chaperone system in the degradation of σ^{32} is that it may translocate σ^{32} to the cytoplasmic membrane, where FtsH exists, and facilitates its degradation. To examine this possibility, we used a soluble form of FtsH, Zip-FtsHΔTM-Myc, in which the N-terminal transmembrane region of FtsH was replaced by a leucine-zipper sequence from the *Saccharomyces cerevisiae* GCN4 protein (Fig. 4A). Zip-FtsHΔTM-Myc localizes in the cytoplasm and possesses activity to degrade cytoplasmic substrates ([30]; Fig. 4B). If the above possibility is correct, Zip-FtsHΔTM-Myc should degrade σ^{32} independently of the DnaK chaperone system. As shown in Fig. 4B, Zip-FtsHΔTM-Myc degraded σ^{32} significantly in ΔftsH cells but not in ΔftsH/ΔdnaK cells. On the other hand, it degraded LpxC, another natural substrate of FtsH [13], in both ΔftsH and ΔftsH/ΔdnaK cells. These results indicate that DnaK is required for the degradation of σ^{32} even if FtsH exists in the cytoplasm and that its requirement is specific for σ^{32}.

Table 1
Stability of σ^{32} variants in vivo[a]

σ^{32}	Host			
	wild-type	ΔftsH	ΔdnaK	ΔdnaJ/ΔcbpA
WT-c-his	<1	83	13	9
Q80R-c-his	<1	158	15	11
Q80N-c-his	<1	114	11	16
F136L-c-his	<1	102	17	25
WT-n-his	<1	154	26	20
L278W-n-his	<1	47	4	6
authentic σ^{32}	<1	105	22	27

[a]Half-lives of proteins (min) are indicated.
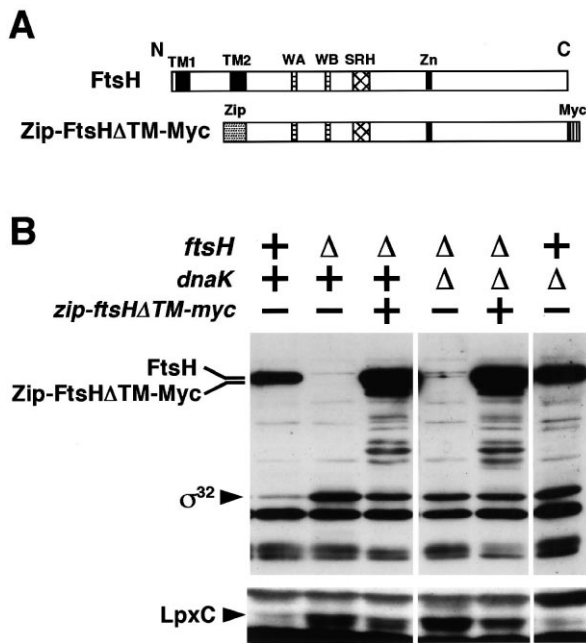
Fig. 4. A: Schematic representation of Zip-FtsHΔTM-Myc. TM, transmembrane region; WA, Walker motif A; WB, Walker motif B; SRH, region conserved among AAA proteins; Zn, Zn^{2+}-binding motif; Myc, C-terminal Myc tag; Zip, leucine-zipper motif of the yeast GCN4 protein. B: Zip-FtsHΔTM-Myc is active for the degradation of σ^{32} in $dnaK^+$ cells but in Δ$dnaK$ cells. AR3291 (Δ$ftsH$; lanes 2 and 3) and AR7051 (Δ$ftsH$/Δ$dnaK$; lanes 4 and 5) cells carrying pUC119 (−) or pSTD430 ($zip-ftsHΔTM-myc$ pUC119; +) were grown in L medium (without IPTG) and aliquots of the cell culture were collected at Klett unit = 50. Cells were harvested and dissolved in SDS sample buffer, then subjected to SDS–PAGE and Western blotting against the mixture of anti-FtsH and anti-σ^{32} antisera or anti-LpxC antiserum. Samples of W3110 (wild-type; lane 1) and AR7051 (Δ$dnaK$; lane 6) were for comparison.

4. Discussion

Rapid turnover of σ^{32} is one of the key regulatory mechanisms in the heat shock response in *E. coli*. Genetic and biochemical evidence indicated that the DnaK chaperone system plays an important role in it, however its precise mechanism has not been elucidated. Several hypotheses have been proposed. A most likely hypothesis is that the sequestration of σ^{32} by the DnaK system from RNAPcore, which is an 'inhibitor' for degradation, is required for the rapid turnover of σ^{32} [20,24]. This hypothesis is based on the findings that addition of RNAPcore interferes with in vitro degradation of σ^{32} by FtsH [24], and that the DnaK chaperone system and RNAPcore share the site in σ^{32} polypeptide for binding [24,36]. Blaszczak et al. [24] speculated that DnaK and DnaJ compete with RNAP for binding to σ^{32} then keep σ^{32} susceptible to degradation. Here we have examined this hypothesis. Our results indicated that mutations affecting the core-binding did not alter the requirement of the DnaK chaperone system in the degradation of σ^{32} in vivo. Thus, we conclude that the core-binding itself is not a major factor affecting the degradation efficiency of σ^{32} in vivo, and the sequestration of σ^{32} from RNAPcore does not explain the requirement of the DnaK chaperone system in the rapid turnover σ^{32} in vivo.

There seems to be a contradiction between our present in vivo results and in vitro results reported previously. This apparent contradiction may be explained as follows. Under steady-state conditions, σ^{70} is more abundant than σ^{32} by two orders of magnitude in the cell. There are also additional σ factors, potential competitors for σ^{32} in binding to RNAPcore in the cell. Thus, a large fraction of σ^{32} would be free from RNAPcore even if it has a higher affinity for RNAPcore than σ^{70}. On the other hand, in vitro reactions contained σ^{70} at most 2-fold higher than σ^{32}, thus it is expected that most σ^{32} proteins bind to RNAPcore and are protected from degradation. Although we cannot exclude the possibility that core-binding contributes in part to the degradation of σ^{32} in vivo, in particular, under the heat-shocked conditions, it is unlikely that the sequestration of σ^{32} is the major role of the DnaK system in degradation in vivo.

Other possibilities for the role of the DnaK chaperone system have also been proposed. The DnaK system may actively present σ^{32} to proteases for degradation [2]. However, attempts to show direct presentation of σ^{32} to FtsH by the DnaK chaperone system in vitro by our and several other groups have been unsuccessful. Alternatively, the DnaK system may translocate σ^{32} to the membrane where FtsH exists. This possibility cannot easily be tested in soluble cell-free systems. We examined the requirement of the DnaK system in the degradation of σ^{32} by a soluble variant of FtsH, and found that the soluble FtsH was inactive for σ^{32} in Δ$dnaK$ cells, excluding the possibility. We also investigated the localization of GFP-tagged σ^{32} in the cell, and observed no difference in localization between Δ$ftsH$ and Δ$dnaK$ cells (data not shown).

Chaperones including DnaK are involved in the degradation of both non-specific and specific substrates by ATP-dependent proteases [1,37]. It has been speculated that chaperones act to keep substrates soluble and protease-accessible. Supporting evidence for this speculation is that RcsA forms insoluble aggregates in Δ$dnaJ$ cells and hence is stabilized [38]. However, this possibility does not seem to fit the case of σ^{32}, as the majority of σ^{32} in Δ$dnaK$ cells are recovered to soluble fraction (data not shown). However, σ^{32} may form small aggregates or complexes with unidentified inhibitors. It is also possible that σ^{32} is in a state of equilibrium of 'tightly folded' and 'loosely folded' forms in vivo. The DnaK chaperone system may alter σ^{32} to a form competent to the FtsH action in vivo, and facilitates its rapid degradation. Further refining of the in vitro assay system is required to address these possibilities.

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