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A kinetic study on antibacterial peptides of *Galleria mellonella* larvae and pupae synthesized in response to challenge with *Escherichia coli* lipopolysaccharide

Badania kinetyczne peptydów odpornościowych gąsienic i poczwarek *Galleria mellonella* immunizowanych lipopolisacharydem *Escherichia coli*

SUMMARY

Inducible antibacterial activity against *Escherichia coli* D31 was identified in the larval and pupal hemolymph of the moth *Galleria mellonella* following a challenge with *Escherichia coli* lipopolysaccharide. The antimicrobial activity appeared 3–6 h after lipopolysaccharide injection, persisted for two days and then gradually disappeared. The kinetics of induction as well as the level of antimicrobial activity differed for three developmental stages studied. The appearance of LPS-induced antibacterial activity clearly correlated with the appearance in hemolymph of at least two defence peptides. Their partial amino acid sequence analysis revealed substantial homology to Gm proline-rich peptide 1 and Gm cecropin-D like peptide. Using a bioautography assay, a possibility of restoration of antibacterial activity of both peptides after electrophoresis in denaturing conditions was demonstrated.

STRESZCZENIE

W hemolimfie gąsienic oraz poczwarek *Galleria mellonella* immunizowanych lipopolisacharydem *E. coli* wykryto aktywność przeciwbakteryjną. Aktywność ta pojawiała się po 3–6 godzinach od podania LPS, utrzymywała się przez około dwie doby, a następnie stopniowo zanikała. Zarówno kinetyka indukcji, jak i poziom aktywności przeciwbakteryjnej były zależne od stadium rozwojowego owada. Najwyższy poziom aktywności przeciwbakteryjnej zanotowano w hemolimfie uzyskanej po 24, 48 i 12–24 godzinach od immunizacji, odpowiednio, gąsienic żerujących, gąsienic wędrujących oraz poczwarek. Stosując trycynową elektroforezę SDS/PAGE oraz metodę bioautografii, wykazano, że aktywność ta jest związana z pojawieniem się w hemolimfie peptydów o masach cząsteczkowych poniżej 6,5 kDa. Analiza sekwencji aminokwasów potwierdziła obecność dwóch

peptydów odpornościowych *G. mellonella* peptydu bogatego w prolinę 1 oraz peptydu podobnego do cekropiny D.

Key words: innate immunity *Galleria mellonella* antibacterial peptides cecropin proline-rich peptide bioautography

List of abbreviations: EWL – hen egg white lysozyme; LPS – lipopolysaccharide; MS/MS – tandem mass spectrometry; PTU – N-phenylthiourea; SDS/PAGE – sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

INTRODUCTION

The insect immune defence is activated by microorganism infection, injury or microbial cell wall components injection and acts through both cellular and humoral responses (Bulet Stöcklin 2005; Hetru et al. 1998). The insect humoral response is manifested by the rapid and transient synthesis of antibacterial and/or antifungal proteins and peptides that are accumulated in the hemolymph. These antimicrobial molecules are mainly cationic peptides with a broad spectrum of activity against Gram-positive and Gram-negative bacteria. A large number of them was biochemically characterized and cloned. On the basis of their molecular structure several types of these natural polypeptide antibiotics were identified. They include α-helical peptides (e.g. cecropins) peptides with intramolecular disulfide bridges (e.g. defensins) multi-domain peptides (e.g. attacins) proline--rich peptides and lysozymes (Boman 1995; Bulet, Stocklin 2005; Hetru et al. 1998; Otvos 2000; Otvos 2002; Raj, Dentino 2002). More than 900 natural peptides were recognized as components of the innate immune systems of eukaryotic organisms. Of that impressive number about 200 were identified in insects (www.bbcm.univ.trieste.it/~tossi/pag1.htm).

In this paper we present the comparative study on the kinetics of induction of the antibacterial peptides in *Galleria mellonella* (Lepidoptera: *Pyralidae*) larvae and pupae challenged with *Escherichia coli* lipopolysaccharide (LPS). We demonstrate that the increase in antimicrobial activity level observed in hemolymph of LPS-challenged *G. mellonella* larvae and to some extent, pupae was correlated with appearing of at least two inducible antibacterial peptides. Using a bioautography assay a possibility of restoration of their antibacterial activity after electrophoresis in denaturing conditions is also presented.

MATERIAL AND METHODS

Insects

Larvae of the greater wax moth *Galleria mellonella* (Lepidoptera: *Pyralidae*) were reared on an artificial diet (12.5% wheat meal, 25% corn meal, 12.5% wheat germ, 12.5% dried milk, 25% honey, 12.4% glycerol and 0.1% dried yeast) at 30°C in the dark, essentially as described (Sehnal 1966) but without bee wax addition. Last instar (seventh) feeding and wandering larvae and 2-day-old pupae were used throughout the study.

Insect immune challenge and hemolymph collection

Immune challenge was performed with *E. coli* LPS (Serotype 026:B6, Sigma) diluted in sterile water. Each *G. mellonella* larvae and pupae was injected with 3 µl of the solution containing 2.5 and 1.25 µg of LPS, respectively. The insects were kept at 30°C in the dark and the hemolymph (20 ml per larvae and 10 ml per pupae) was collected after time indicated in the text. Prior to hemolymph collection, the insects were chilled for 15 min at 4°C. Hemolymph samples were obtained by puncturing larval abdomen and pupal head with a sterile needle. Out-flowing hemolymph was

immediately transferred into sterile and chilled Eppendorf tubes (samples from three insects were combined) containing a few crystals of phenylthiourea (PTU) to prevent melanisation. The hemocyte-free hemolymph was obtained by centrifugation at 200×*g* for 5 min and subsequently at 20000×*g* for 15 min at 4°C. Pooled supernatants were used immediately or stored at -20°C until used.

Acidic/methanolic extraction of small proteins and peptides

Peptides of antibacterial activity were partially purified from the hemocyte-free hemolymph by acidic/methanolic extraction method adapted from Schoofs et al. (Schoofs et al. 1990) and described previously (Cytryńska et al. 2007). Briefly, the hemolymph was diluted ten times with the extraction solution consisting of methanol/acetic acid, glacial/water (90:1:9) and mixed thoroughly. Precipitated proteins were pelleted by centrifugation at 20000×*g* for 30 minutes at 4°C. The obtained supernatant containing mainly proteins of M_r below 30 kDa was collected, vacuum dried and the pellet was stored at -20°C until needed. Before use it was dissolved in appropriate volume of water (usually the water volume was 2/3 of the initial hemocyte-free hemolymph volume). The protein concentration was 1.5–2.0 mg/ml.

Antibacterial activity assay

Diffusion well assay

For antibacterial activity tests, a LPS defective, streptomycin and ampicillin resistant mutant of *E. coli* K21, strain D31 was used (Boman et al. 1974). The presence of antibacterial activity in the hemolymph and acidic/methanolic extracts of hemolymph was detected by growth inhibition zone assay using solid agar plates containing viable *E. coli* D31 cells as described (Hoffmann et al. 1981). To improve the sensitivity of the method hen egg white lysozyme (EWL) in the concentration of 2.5 mg/ml of the medium was added (Chalk et al. 1994). Each well on the Petri dish was filled with 5 µl of hemolymph four times diluted with sterile water or 5 µl (10 µg of protein) of dissolved partially purified peptides. The agar plates were then incubated at 37°C for 24 h. The diameters of *E. coli* D31 growth inhibition zones were measured. The level of antimicrobial activity was calculated using the algorithm described by Hultmark (Hultmark et al. 1982). For evaluation of antibacterial activity synthetic cecropin B of *Hyalophora cecropia* (Sigma) was used as a standard.

SDS gels overlay method (bioautography)

Detection of antibacterial activity after SDS/PAGE and subsequent renaturation of polypeptides in the gels was performed as described previously (Cytryńska et al. 2001). Briefly, after resolution of proteins, the gels were washed for 30 min in 2.5% Triton X-100 (Bio Rad) for SDS removal. Then polypeptides were renatured in 50 mM Tris-HCl pH 7.5 and subsequently in Luria broth, each step for 30 min. The gels were overlaid with *E. coli* D31 indicator strain suspended in nutrient agar containing 2.5 mg/ml of EWL and incubated for 6–12 h at 37°C. After that time, zones of bacterial growth inhibition were observed. As the control peptide synthetic cecropin B (Sigma) in the amount of 1 µg was used.

Identification of peptides

Identification of peptides from immune *G. mellonella* hemolymph was performed at the Institute of Biochemistry and Biophysics (Warsaw, Poland) by MS/MS method (Tandem Mass Spectrometry) using the Q-Tof1 spectrometer (Micromass) equipped with nano-HPLC column (LC Packings).

Proteins and peptides obtained from the *G. mellonella* hemolymph after acidic/methanolic extraction were subjected to tricine SDS/PAGE (16.5% T, 3% C) according to Schägger and von Jagow (Schägger, von Jagow 1987). Then polypeptide bands in the gel were stained with Coomassie

Brilliant Blue G-250. Appropriate peptide bands were cut out from the gel and after trypsin digestion examined by mass spectrometry.

Other methods

Polyacrylamide gel electrophoresis of proteins was performed by 13.8% glycine SDS/PAGE according to Laemmli (Laemmli 1970). For better resolution of proteins below 30 kDa and peptides, tricine SDS/PAGE (16.5% T, 3% C) as described by Schägger and von Jagow was used (Schägger, von Jagow 1987).

The protein concentration was estimated by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976).

RESULTS AND DISCUSSION

It is known from the earlier research that in *G. mellonella* larvae and pupae, antibacterial activity is induced after injection of bacteria or bacterial cell wall components into the hemocoel and can be detected in hemolymph of immune challenged insects (Hoffmann et al. 1981; Jarosz 1993). In this work, *E. coli* LPS was used for the kinetic studies on antibacterial activity induction in *G. mellonella* last instar feeding and wandering larvae and 2-day-old pupae. In all the three developmental stages studied, antibacterial activity against *E. coli* was detected as early as 6 h after immune challenge (Fig. 1). The peak of anti-*E. coli* activity was

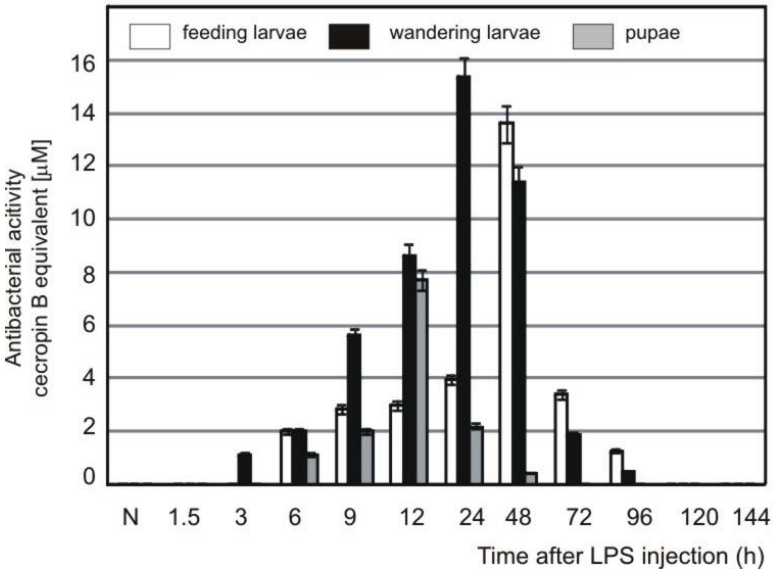

Fig. 1. Antibacterial activity in hemolymph of *G. mellonella* larvae and pupae challenged with *E. coli* LPS. Anti-*E. coli* activity was detected using the diffusion well assay. Hemolymph samples collected at different time intervals after LPS injection were tested on Petri agar plates containing 2.5 mg/ml of EWL. The values were calculated from three independent experiments and were presented as ±SD. N – non-immune hemolymph

observed 24 h, 48 h and 12–24 h after LPS injection in feeding larvae, wandering larvae and pupae hemolymph, respectively, and then gradually disappeared over a few days. The highest level of antibacterial activity was detected in the hemolymph of wandering larvae and the concentration of antibacterial peptides was calculated as 15.5 μM in the terms of cecropin B activity. In the pupae hemolymph the anti-*E. coli* activity level was, in general, lower and the concentration of antibacterial peptides was calculated as 7.8 μM of cecropin B for 12 h after LPS challenge (Fig. 1).

In the following experiments, samples of the acidic/methanolic extracts obtained from hemolymph of *G. mellonella* immune-challenged larvae and pupae were analysed by tricine SDS/PAGE which is routinely used for separation of low molecular weight proteins and peptides. The enhanced amount of a 15 kDa protein appeared on the stained gels in larval hemolymph collected 12–72 h after immunization (Fig. 2). The molecular mass of this protein corresponded to lysozyme, which is consistent with the earlier observations, indicating lysozyme to be a prominent component of the immune defence in many insect species (Hultmark, 1996).

From Figure 2 it can be seen that two peptide bands corresponding to M_r of 4.2 kDa and 3.5 kDa appeared transiently in *G. mellonella* hemolymph 12–72 h after immune challenge. Their appearance in *G. mellonella* immune hemolymph correlated with the peak of anti-*E. coli* activity detected in larval and, to some extent, pupal hemolymph by using diffusion well assay (Fig. 1). To confirm that inducible hemolymph peptides were responsible for detected anti-*E. coli* activity, we performed bioautography study by using SDS gels overlay method which allowed to detect antibacterial peptides activity *in situ* (Cytryńska et al. 2001). These experiments were performed using cell-free hemolymph collected 9–96 h after LPS injection and compared with extracts obtained from hemolymph collected 24 h after immune challenge. As it can be seen in Figure 3A, in the case of all the challenged samples resolved by glycine SDS/PAGE, a single *E. coli* D31 growth inhibition zone corresponding approximately to the molecular mass of cecropin B used as molecular weight and activity marker, was identified. In hemolymph extracts resolved by tricine SDS/PAGE, two growth inhibition zones corresponding to 4.2 kDa and 3.5 kDa were visible (Fig. 3B). As for not challenged samples, such zones were not observed (data not shown).

The molecular mass of additional peptide bands appearing in the immune hemolymph indicated antibacterial peptides induced by LPS injection. To identify both peptides, tandem mass spectrometry (MS/MS) was used. Proteins and peptides of the extract obtained from hemolymph of *G. mellonella* larvae collected 24 h after immune challenge were separated by tricine SDS/PAGE and briefly stained with Coomassie Brilliant Blue G-250. Two peptide bands of M_r 4.2 kDa (peptide band A) and 3.5 kDa (peptide band B) were cut out separately from the

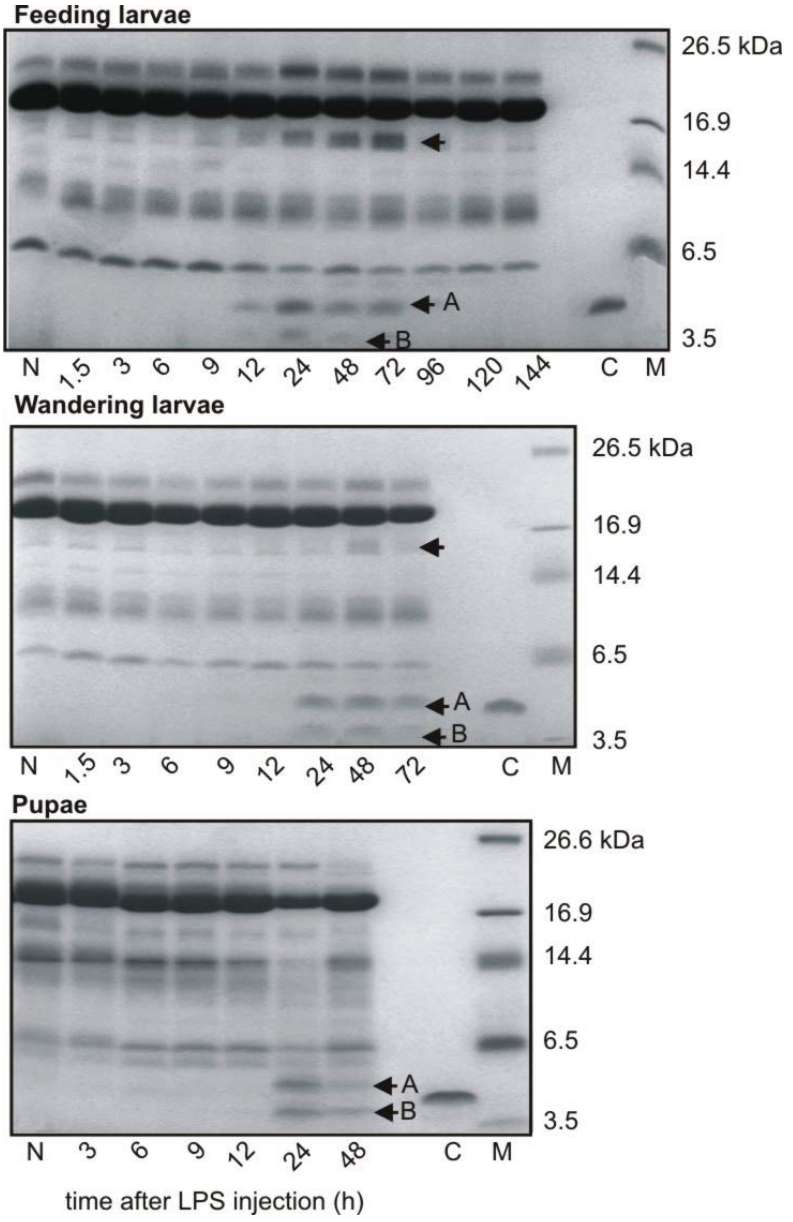

Fig. 2. Tricine SDS/PAGE of polypeptides obtained from *G. mellonella* hemolymph by acidic/methanolic extraction. Proteins were extracted from non-immune (N) and immune hemolymph collected after LPS injection at the time indicated and resolved in 16.5% 15×15 cm tricine SDS gels. Each line on the gel contained 20 μg of proteins. Two additional peptide bands, A and B, appearing in immune hemolymph and protein band corresponding to lysozyme were marked by arrows. The presented electrophoretograms were representative for three independent experiments. M – molecular weight standards; C – cecropin B (1 μg)

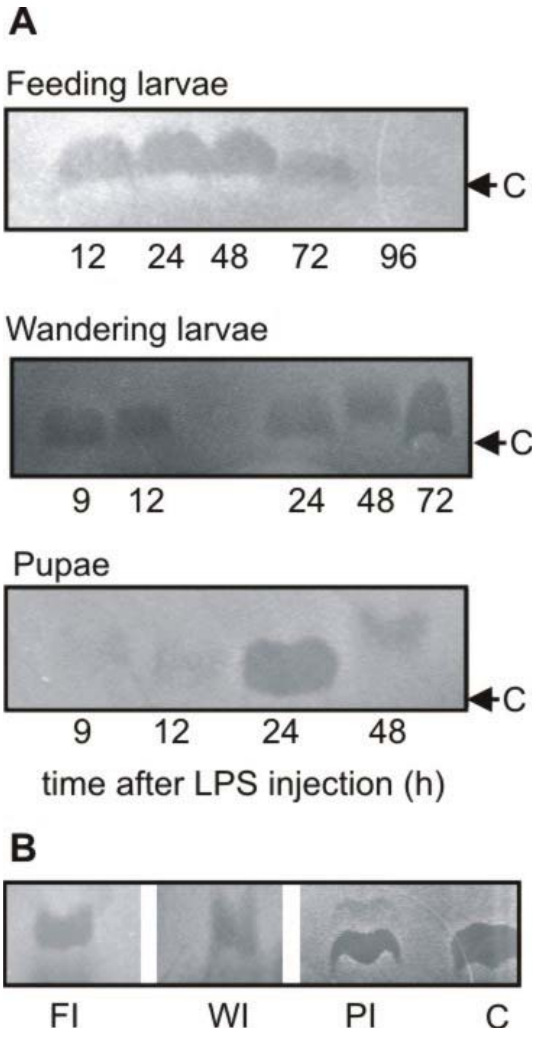

Fig. 3. Bioautography of renatured *G. mellonella* antibacterial peptides separated by SDS/PAGE. Immune hemolymph samples (100 µg of total protein) collected after LPS injection at the time indicated were resolved in mini gels 8×5.5 cm (width×length) by 13.8% glycine SDS/PAGE (A) and acidic/methanolic extract samples (30 µg of total protein) obtained from hemolymph collected 24 h after immune challenge were resolved by 16.5% tricine SDS/PAGE (B). After renaturation, the gels were overlaid with *E. coli* D31 in nutrient agar containing 2.5 mg/ml of EWL. One microgram of cecropin B (C) was used as a control. The localization of bacteria growth inhibition zone caused by cecropin B in SDS overlay method presented in part A was marked by the arrows. FI – extract from immune hemolymph of feeding larvae; WI – extract from immune hemolymph of wandering larvae; PI – extract from pupae immune hemolymph. Fragments of the gels visualizing bacteria growth inhibition zones, corresponding approximately to cecropin B molecular weight were presented. For gels images acquisition and documentation, the video image analyser Gel Doc 2000 with Quantity One Software (Bio Rad) was used

Table 1. Amino acid sequence analysis of G. *mellonella* A and B peptide bands by MS/MS method

Peptide band	Sequenced tryptic peptide fragments	Tryptic peptide fragments identified by molecular mass
A	DIQIPGIK DIIIPNWNPNVR	TQPWQR DIQIPGIKKPTHR
B	IRDAIISAAPAVETLAQAQK	ENFFKEIER ENFFK AGQRIR

gel and subjected to amino acid analysis using the MS/MS method after tryptic digestion. The obtained results, presented in Table 1 and Figure 4, revealed the presence of Gm proline-rich peptide 1 and Gm cecropin D-like peptide in band A and B, respectively. The peptides, along with other defence peptides have been recently described in immune *G. mellonella* larval hemolymph (Brown et al. 2009; Cytryńska et al. 2007).

We presented a comparative study on the kinetics of induction of the antibacterial activity in *G. mellonella* larvae and pupae after LPS challenge. The highest level of anti-*E. coli* activity was observed in the larvae hemolymph (15.5 μM in the terms of cecropin B activity). The kinetics of induction as well as the level of antimicrobial activity was dependent on the developmental stage of *G. mellonella*,

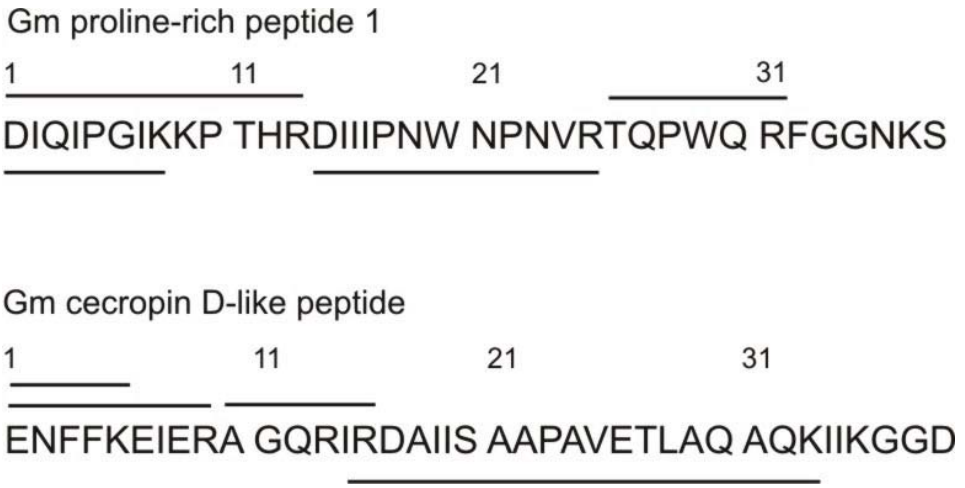

Fig. 4. Comparison of antibacterial peptide amino acid sequences obtained in this study with known *G. mellonella* peptides. Tryptic peptides identified in this study by partial sequencing were underlined. The sequences marked with a line above were identified by molecular mass determination

most probably reflecting physiological differences between feeding larvae, wandering larvae and pupae.

The increase in antimicrobial activity level clearly correlated with the presence in the immune hemolymph of at least two inducible antibacterial peptides of M_r 4.2 kDa and 3.5 kDa. They were identified as Gm proline-rich peptide 1 and Gm cecropin D-like peptide and their anti-*E. coli* activity was demonstrated using SDS gels overlay method. Our results indicated that both identified peptides, surely among a large number of other antimicrobial molecules, were responsible for antibacterial activity in the hemolymph of LPS-challenged larvae and pupae of *G. mellonella*.

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