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Post-transcriptional Regulation of Genes Encoding Anti-microbial Peptides in *Drosophila*[*] [S]

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Secretion of antimicrobial peptides (AMPep) is a central defense mechanism used by invertebrates to combat infections. In *Drosophila* the synthesis of these peptides is a highly regulated process allowing their rapid release in the hemolymph upon contact with pathogens and the arrest of their production after pathogen clearance. We observed that AMPep genes have either a transient or sustained expression profile in S2 D*rosophila* cells treated with peptidoglycan. Moreover, AMPep genes containing AU-rich elements (ARE) in their 3′-untranslated region (UTR) are subject to a post-transcriptional control affecting mRNA stability, thereby contributing to their transient expression profile. Cecropin A1 (CecA1) constitutes the prototype of this latter class of AMPeps. CecA1 mRNA bears in its 3′-UTR an ARE similar to class II AREs found in several short-lived mammalian mRNAs. In response to immune deficiency cascade signaling activated by Gram-negative peptidoglycans, CecA1 mRNA is transiently stabilized and subsequently submitted to deadenylation and decay mediated by the ARE present in its 3′-UTR. The functionality of CecA1 ARE relies on its ability to recruit TIS11 protein, which accelerates CecA1 mRNA deadenylation and decay. Moreover, we observed that CecA1 mRNA deadenylation is a biphasic process. Whereas early deadenylation is independent of TIS11, the later deadenylation phase depends on TIS11 and is mediated by CAF1 deadenylase. We also report that in contrast to tristetraprolin, its mammalian homolog, TIS11, is constitutively expressed in S2 cells and accumulates in cytoplasmic foci distinct from processing bodies, suggesting that the *Drosophila* ARE-mediated mRNA deadenylation and decay mechanism is markedly different in invertebrates and mammals.

Activation of the immune response by recognition of pathogen-associated molecular patterns is a conserved mechanism among all metazoans. These molecular signatures present in pathogens but absent in the host are recognized by extra or intracellular receptors and activate downstream signaling cascades (1). In mammals, these primary events contribute to the activation of both innate and adaptive arms of the immune response, whereas invertebrates solely rely on innate immunity to eliminate pathogens.

In *Drosophila*, an important aspect of the immune response triggered by pathogen-associated molecular pattern recognition is the secretion in the hemolymph of a large variety of antimicrobial peptides (AMPeps).[6] AMPeps are cationic molecules supposedly active against pathogen membranes. These peptides are classified in three groups according to their main biological targets: Gram-positive bacteria (defensin), Gram-negative bacteria (cecropins, drosocin, attacins, diptericin), and fungi (drosomycin, metchnikowin) (2, 3). Pathogen recognition in *Drosophila* activates two major signaling pathways. Molecular signatures derived from Gram-positive bacteria and fungi lead to the activation of the Toll pathway, whereas peptidoglycans from Gram-negative microorganisms specifically signal through the immune deficiency cascade (Imd) (4, 5). The Toll receptor (TLR) is the ancestor of the mammalian TLR receptor family (6). It has been demonstrated that engagement of Toll induces the recruitment of Myd88 and Tube adaptors leading to the activation of Pelle kinase. Ultimately, this pathway leads to the activation of Dif and Dorsal, two NF-κB homologs. The inactivation of several members of the Toll cascade clearly demonstrated the relevance of this pathway in *Drosophila* immunity against Gram-positive bacteria and fungi (7, 8).

Activation of the Imd pathway is initiated by the recognition of monomers or polymers of Gram-negative peptidoglycans by members of the peptidoglycan receptor family. These receptors recruit the Imd protein, a homologue of the death domain mammalian protein RIP. Therefore, the Imd pathway is considered as an ancestor of the mammalian tumor necrosis factor receptor pathway. Imd signaling is branched and can lead both to the activation of the mitogen-activated protein kinase kinase kinase TAK1 and to the recruitment of the FADD (FAS-associated death domain protein) death domain protein linked to the activation of Dredd caspase. Ultimately, Imd signaling directs the proteolytic activation of Relish, another member of the NF-κB family of transcription factors. Moreover, TAK1

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[6] The abbreviations used are: AMPep, antimicrobial peptide; Imd, immune deficiency; UTR, untranslated region; RT, reverse transcription; GFP, green fluorescent protein; dsRNA, double-stranded RNA; CecA1, cecropin A1; ARE, AU-rich element; TTP, tristetraprolin; JNK, Jun N-terminal kinase.

contributes to the activation of the Jun N-terminal kinase (JNK) cascade (2).

Microarray analysis of gene transcription in the immune-responsive S2 *Drosophila* cell line upon stimulation with different pathogen-associated molecular patterns has demonstrated that Imd signaling leads to the activation of a large number of genes involved in immune defenses (9, 10). In contrast, activation of the Toll pathway by living *Candida albicans* generates a more restricted transcriptional signature (11). Park *et al.* (10) have shown that Imd activation in S2 cells induces different temporal clusters of gene expression. A first group of transiently expressed genes displays a maximal expression about two h after activation, which rapidly declines within 4 h. It has been demonstrated that these genes are under the control of the JNK pathway. Indeed, inhibition of JNK expression by RNAi abolishes the expression of this first cluster of gene in response to Imd pathway activation. The second cluster of genes induced by Imd signaling displays a sustained expression during 4–6 h. Several genes encoding anti-microbial peptides have been identified in this group (10).

In this study we report that some AMPep genes have a transient expression profile in S2 cells treated with *Escherichia coli* peptidoglycan, thereby demonstrating that these AMPeps genes belong to the first cluster of genes transiently expressed in response to Imd signaling. However, their transient expression is independent from the JNK cascade but, rather, relies on a post-transcriptional control affecting mRNA stability. Cecropin A1 (CecA1) constitutes the prototype of this transiently expressed AMPep. CecA1 mRNA is short-lived due to the presence of an AU-rich element (ARE) present in its 3′-UTR. This element recruits TIS11 protein, thereby accelerating mRNA deadenylation and decay. The analysis of CecA1 mRNA poly(A) status revealed that deadenylation is a biphasic process. Whereas the early deadenylation phase is independent of TIS11, the later deadenylation step depends on TIS11 and is mediated by the CAF1 deadenylase. In mammals, the production of tristetraprolin (TTP), an homolog of TIS11, is activated at the transcriptional level by pathogen-associated molecular pattern stimulation of immune cells. TTP imposes a negative feedback loop to control ARE mRNA half-lives (12). Here, we observed that *tis11* gene is constitutively expressed in S2 cells, suggesting that the ARE decay mechanism is regulated differently in invertebrates and mammals. Finally, we observed that TIS11 accumulates in cytoplasmic foci distinct from P-bodies, suggesting that this *Drosophila* protein localizes differently as compared with TTP, its mammalian counterpart.

Altogether, our data unravel the existence of a new post-transcriptional regulatory pathway controlling the expression of several AMPep genes in *Drosophila*. By mediating deadenylation and decay of ARE-containing mRNAs, this pathway contributes to the transient expression of AMPep genes and thereby participates in the regulation of the innate immune response.

EXPERIMENTAL PROCEDURES

Reagents—E. coli peptidoglycan was purchased from Invivogen (San Diego, CA). Actinomycin D, clasto-lactacystin β-lactone, and DNA oligonucleotides were purchased from Sigma-Aldrich.

Plasmids—The luciferase reporter genes were constructed as follows. The luciferase coding sequence was PCR-amplified with the following oligonucleotides: forward, GAATTCACCATGGAAGACGCCAAAA; reverse, GAATTCGGATCCTTACACGGCGATCTTTCCGCC. The PCR fragment was digested with EcoRI and inserted in the pMT plasmid in which the SV40 polyadenylation signal had been deleted (Invitrogen). The CecA1 3′-UTR was amplified by RT-PCR with the oligonucleotides CecA1 forward, 5′-GGATCCAAGCTTCCACGATGATTATTTATAA-3′, and CecA1 reverse, 5′-GGATCCTTCTTCTTTAAATTTTTAAAATT-3′. The DNA fragment was cloned in the BamHI site of the pMT-luciferase vector. DNA sequence corresponding to the ARE-deleted CecA1 3′-UTR was obtained by *in vitro* hybridization of oligodeoxyribonucleotides corresponding to the sequence 5′-GATCCAAGCTTCCACGATGACCACGATGAAGATCCTGTTGCTCCCTGTAAATAAAACAATTTTAAAAATTTAAAGAA-3′. The fragment was cloned in the BamHI site of the pMT luciferase vector. The GFP and GFP-TIS11 expression constructs were obtained by PCR amplification of the GFP sequence and cloning in the XbaI site of the pMT vector. The complete TIS11-coding sequence was amplified by RT-PCR using the oligonucleotides forward, 5′-GGATCCATGTCTGCTGATATTCTGCA-3′, and reverse, 5′-GAATTCTTTAGATCCCAAATTGGACT-3′ and inserted in the pMT-GFP vector.

Plasmids for RNAi experiments were obtained by RT-PCR amplification of ~500-bp fragments of *Drosophila* target genes cloned in the pBluescript vector. All DNA constructs were verified by DNA sequencing (Genome Express, Meylan, France). Previously described GFP-Dcp1 and GFP-GW182 constructs (13) were kindly provided by E. Izzauralde.

Antibodies—Anti-GFP antibody was purchased from Roche Applied Science. Anti-TIS11 antibody was obtained by immunization of New Zealand White rabbits with the TIS11 C-terminal peptide (LSSGVEAYQQQSNLGL) coupled to keyhole limpet hemocyanin. Twelve days after the third immunization, total exsanguination was performed by cardiac puncture. For immunofluorescence experiments, anti-TIS11 antibody was purified by ammonium sulfate precipitation and affinity chromatography on agarose beads coupled with the immunization peptide.

Cell Culture, Transfection, and RNAi Experiments—Drosophila S2 cells were originally purchased from Invitrogen. Cells were maintained in serum-free medium (Express Five, Invitrogen) at room temperature as described (14). Transfections were performed with FuGENE HD according to the manufacturer's instructions (Roche Applied Science). When indicated, transcription driven by metallothionein promoter was induced by treating the cells with $CuSO_4$ (0.5 mM) for the indicated times. Peptidoglycan was added to the cells at a concentration of 7 μg/ml. For mRNA half-life measurements, transcription was blocked by actinomycin D (5 μg/ml), and the cells were harvested at the indicated time points. The proteasome inhibitor clasto-lactacystin b-lactone was used at the final concentration of 2.5 μM. Double-strand RNA preparations and S2

cell treatments for RNAi were performed as previously described (15).

RNA Analysis—Total RNA was purified using Tri reagent (MBI). For each sample, 8 μg of total RNA were loaded on 1.6, 2.0, or 2.5% agarose gels and transferred to a Hybond-N membrane (Amersham Biosciences). The blots were hybridized with RNA probes transcribed with ^{32}P-labeled UTP (PerkinElmer Life Sciences). RNA probe complementary to rp49 sequence (encoding ribosomal protein L32) was used to normalize RNA loading. Quantification of the radioactive signals was performed with a PhosphorImager (GE Healthcare).

In vitro deadenylated RNA was generated by hybridization of total RNA with oligo(dT) (18–21-mer) at 37 °C for 30 min in the presence of 1 unit of RNase H. RNase H-treated and untreated RNA were separated on 2.5% agarose gel and analyzed by Northern blot. RT-PCR was performed with the Thermoscript reverse transcriptase (Invitrogen) and Platinum Taq polymerase (Invitrogen) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay and Protein Analysis—Analysis of RNA-protein interaction by electrophoretic mobility shift assay and Western blot analysis was performed exactly as described (16).

Fluorescence Microscopy—Transfected S2 cells were seeded on glass coverslips treated with concanavalin A for 2 h. Samples were washed twice with phosphate-buffered saline (PBS), fixed with paraformaldehyde 4% for 10 min, rinsed three times in PBS for 10 min, permeabilized with methanol 100% at −20 °C for 10 min, and rinsed three times in PBS for 10 min. After fixation, samples were blocked in 10% bovine serum albumin for 30 min, incubated with primary antibody diluted in 0.1% Tween 20 (1:50) at room temperature for 1 h, rinsed 3 times in 0.1% Tween 20 for 10 min, incubated with secondary antibody (Alexa594-conjugated donkey anti-rabbit IgG, Molecular Probes) diluted in 0.1% Tween 20 (1:2000) for 1 h at room temperature, and rinsed 3 times in 0.1% Tween 20 for 10 min. The coverslips were rinsed with water and mounted with fluorescent mounting medium (Dako) supplemented with 4′,6-diamidino-2-phenylindole (100 pg/ml) and sealed with nail polish. Image stacks were acquired on the z axis according to Nyquist criteria using a Zeiss Axio Observer inverted microscope and 63× (n = 1.40) or 100× (n = 1.46) apochromat objectives. Raw images were deconvolved with the constrained iterative algorithm of the Axiovision software.

RESULTS

Several AMPep-coding Genes Are Transiently Induced in S2 Cells upon Peptidoglycan Stimulation—The embryo-derived S2 cell line is a widely used *Drosophila* cellular model that has macrophage-like properties such as phagocytosis and the ability to secrete AMPeps in response to several immune activators (Refs. 17, 18, and 19 and references therein). We, therefore, used S2 cells stimulated by peptidoglycan to study the expression profile of AMPep coding genes in response to Imd pathway activation. Total RNA was isolated from S2 cells treated by peptidoglycan at different time points, and the expression of several AMPep coding genes was analyzed by Northern blot. As previously described (10), we observed that the activation of the

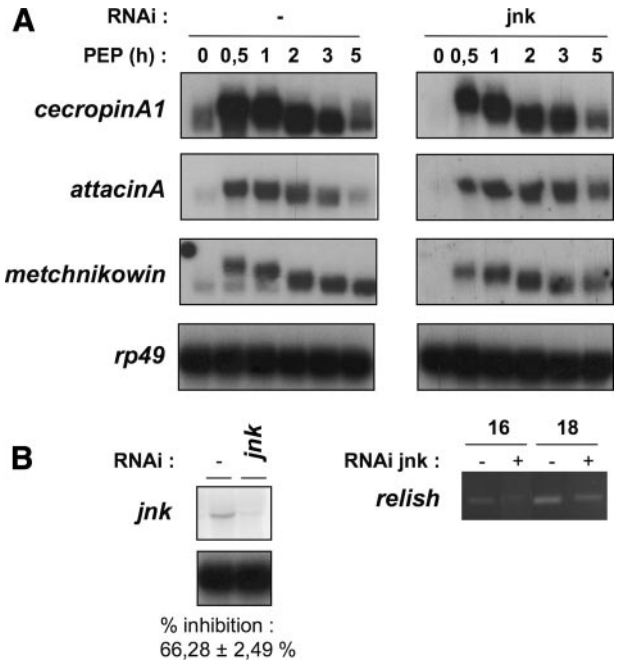

FIGURE 1. **Sustained or transient expression of AMPep coding genes in response to Imd signaling.** *A*, S2 cells were pretreated or not with double-stranded RNA directed against *basket* gene (encoding JNK) for 3 days before treatment with peptidoglycan (*PEP*) for the indicated times. Total RNA was extracted and analyzed by Northern blot using the indicated riboprobes for hybridization. Detection of rp49 mRNA was performed to verify equal loading of total mRNA on gels. *B*, knock-down of JNK was confirmed by Northern blot (*jnk*) or RT-PCR (*relish*) stopped after 16 and 18 cycles. The rate of JNK inhibition was determined by quantification of Northern blot performed on three independent experiments. The mean value ± S.D. is indicated.

Imd pathway in S2 cells led to a rapid and prolonged accumulation of some AMPep mRNAs. Indeed, mRNA encoding Metchnikowin peptide accumulated at high levels 30 min after peptidoglycan addition to the cell culture and remained abundant up to 5 h of stimulation (Fig. 1*A*, *left panel*). In contrast, cecropin A1 and attacin A mRNAs rapidly accumulated upon peptidoglycan stimulation but markedly decreased 3–5 h thereafter (Fig. 1*A*). To confirm that the Toll pathway was not activated in our experimental conditions, we verified that defensin mRNA was not induced in response to peptidoglycan activation of S2 cells (not shown) (2).

A similar transient expression pattern was reported for a group of genes activated by the Imd pathway (9, 10). The transcriptional activation of these genes requires JNK signaling which is down-regulated after several hours of Imd activation. The down-regulation of JNK activity results in the clearance of the accumulated mRNAs after several hours of continuous stimulation. Therefore, we investigated the role of JNK in the peptidoglycan-induced expression of AMPeps by a RNA interference approach (see "Experimental Procedures" and Fig. 1*B*, *left panel*). We observed that JNK inactivation did not interfere with the induction of CecA1, attacin A, and metchnikowin in response to peptidoglycan (Fig. 1*A*, *right panel*) but inhibited the induction of rel as previously described (Fig. 1*B*, *right panel*) (10). These results demonstrate that the transcriptional activation of these AMPep genes is independent of JNK signaling and corroborate previous observations showing that the transcrip-

tional activation of attacin A depends on relish activator but is independent of JNK (10).

Of note, prolonged stimulation of S2 cells with peptidoglycan induces a size reduction of AMPep mRNAs (Fig. 1*A*). This size reduction was observed for all AMPep mRNAs tested, although the effect was more easily detected for short mRNAs such as CecA1 and metchnikowin. These observations suggest that AMPep mRNAs could be rapidly deadenylated after synthesis.

Different Half-lives of AMPep mRNAs in Peptidoglycan-stimulated S2 Cells—To determine whether the transient expression of certain AMPep genes relied on a post-transcriptional regulatory mechanism, we assessed the stability of the corresponding mRNAs. S2 cells were stimulated with peptidoglycan for 3 h before transcriptional blockade with actinomycin D. Total RNA was extracted at different time points after the addition of actinomycin D and was used for a Northern blot analysis to assay AMPep mRNA decay rate. This experiment revealed that CecA1 mRNA was rapidly degraded with an approximate half-life of 200 min (Fig. 2, *A* and *C*). In contrast, attacin A and metchnikowin mRNAs were significantly more stable. Although a precise determination of their half-life was not possible due to actinomycin D toxicity upon prolonged treatment, our experiments revealed that more than 50% of these mRNAs was still detectable 240 min after transcriptional blockade (Fig. 2, *A* and *C*).

Interestingly, several AMPep mRNAs contain sequences rich in adenine and uridine in their 3′-UTR, which are similar to functional AREs found in unstable mammalian mRNAs. In mammals, these sequence elements mediate mRNA instability by recruiting TTP or BRF1 proteins (20, 21). Jing *et al.* (22) demonstrated that the product of *tis11* gene mediated the accelerated degradation of reporter mRNAs bearing mammalian AREs in their 3′-UTR. Therefore, we tested the effect of *tis11* inactivation on the stability of AMPep mRNAs. Cells were treated for 3 days with double-stranded RNA (dsRNA) targeting the *tis11* sequence before stimulation by peptidoglycan and transcriptional blockade by actinomycin D. The efficient inactivation of *tis11* was confirmed by Northern blot analysis of *tis11* mRNA (Fig. 2*B*). We observed that *tis11* inactivation induced a significant stabilization of CecA1 mRNA (half-life >240 min). Again, actinomycin D toxicity after 5–6 h of treatment precluded mRNA half-life determination for stable mRNAs. However, we observed that the amount of attacin A mRNA remaining in the cells after 2 and 4 h of treatment was increased by 20% upon *tis11* inactivation (Fig. 2*A*, compare *left* and *right panels*). This result indicates that TIS11 also exerts a moderate but detectable effect on attacin A mRNA half-life. Of note, the stability of metchnikowin mRNA remained unaffected by RNAi-induced *tis11* inhibition.

Altogether, our results indicate that AMPep genes such as CecA1 and attacin are transiently expressed in response to peptidoglycan stimulation. These transient expression patterns are modulated at the post-transcriptional level by TIS11 protein.

TIS11 Binds to CecA1 mRNA 3′-UTR and Regulates mRNA Stability via a Functional ARE—TIS11 protein is highly similar to TTP, BRF-1, and BRF-2 mammalian ARE-binding proteins. This conservation is particularly important in the region containing two CCCH-type zinc finger motifs (68.6% amino acid identity; data not shown) which encounter for the specific binding to AU-rich sequences present in the 3′-UTR of several mRNAs (23). Therefore, we tested the ability of a GFP-TIS11 fusion protein to specifically bind to CecA1 mRNA 3′-UTR. S2 cells were transfected with GFP or GFP-TIS11 coding constructs. Protein extracts were prepared from transfected cells and incubated with a ^{32}P-labeled RNA probe corresponding either to the 3′-UTR of CecA1 mRNA or to the antisense sequence of CecA1 messenger RNA. The incubation was performed in the presence or the absence of a GFP-specific antibody and with RNase T1 to degrade the unprotected RNA probe. RNA-protein complexes were resolved by electrophoretic mobility shift assay. As shown in Fig. 3*A*, the migration of CecA1 3′-UTR RNA probe was specifically retarded upon incubation with protein extract derived from cells transfected with the GFP-TIS11 construct but not with the GFP control construct. Furthermore, the addition of GFP antibody in the reaction induced a supershift of the RNA-protein complex confirming the presence of the GFP moiety in the complex. No complex could be detected with CecA1 antisense RNA probe, thereby demonstrating the specificity of GFP-TIS11 association to CecA1 3′-UTR.

The 3′-UTR of CecA1 mRNA contains several repeats of the pentanucleotide AUUUA which constitutes the core motif of AREs in vertebrates. This sequence element is present in the majority of mRNA targets destabilized by TTP and BRF1 proteins, albeit several non AU-rich sequences can directly interact with TTP (24). Reporter genes containing AREs from mammalian origin are efficiently degraded in *Drosophila* cells (22). However, Bönisch *et al.* (25) recently reported that the short half-life of *Drosophila* mRNA encoding HSP70 is not influenced by the AU-rich element present in its 3′-UTR. Thus, the role of AREs present in *Drosophila* mRNAs has not been clearly established so far. To test the ability of the ARE present in CecA1 mRNA 3′-UTR to induce a TIS11-dependent destabilization, we generated two reporter genes in which the luciferase coding sequence was placed under the control of the metallothionein promoter and flanked by CecA1 3′-UTR containing or not the ARE. These constructs were transfected in S2 cells either left untreated or preincubated with *tis11* dsRNA. The transcription of the reporter genes was induced by incubation of the cells with copper sulfate, and the half-life of the mRNAs was determined by Northern blot as described above. We observed that the reporter mRNA bearing CecA1 ARE was short-lived ($t_{1/2}$ = 78 min) and was markedly stabilized upon *tis11* inhibition ($t_{1/2}$ > 150 min). In contrast, the reporter mRNA bearing a 3′-UTR lacking the ARE (supplemental Fig. 1*A*) was significantly more stable ($t_{1/2}$ > 150 min), and its half-life was not significantly modified by *tis11* inhibition (Fig. 3, *B* and *C*).

In mammals previous studies have shown that AUUUA pentamers are not sufficient to induce mRNA destabilization and that functional ARE must contain U residues flanking the AUUUA pentamers (26, 27). We observed that the ARE motif present in the CecA1 mRNA contains several AUUUA motifs flanked by uridine residues (supplemental Fig. 1*B*). To define more precisely the destabilizing motif within the CecA1 ARE, we generated a reporter gene in which all the uridine residues flanking AUUUA pentamers in CecA1 mRNA 3′-UTR were

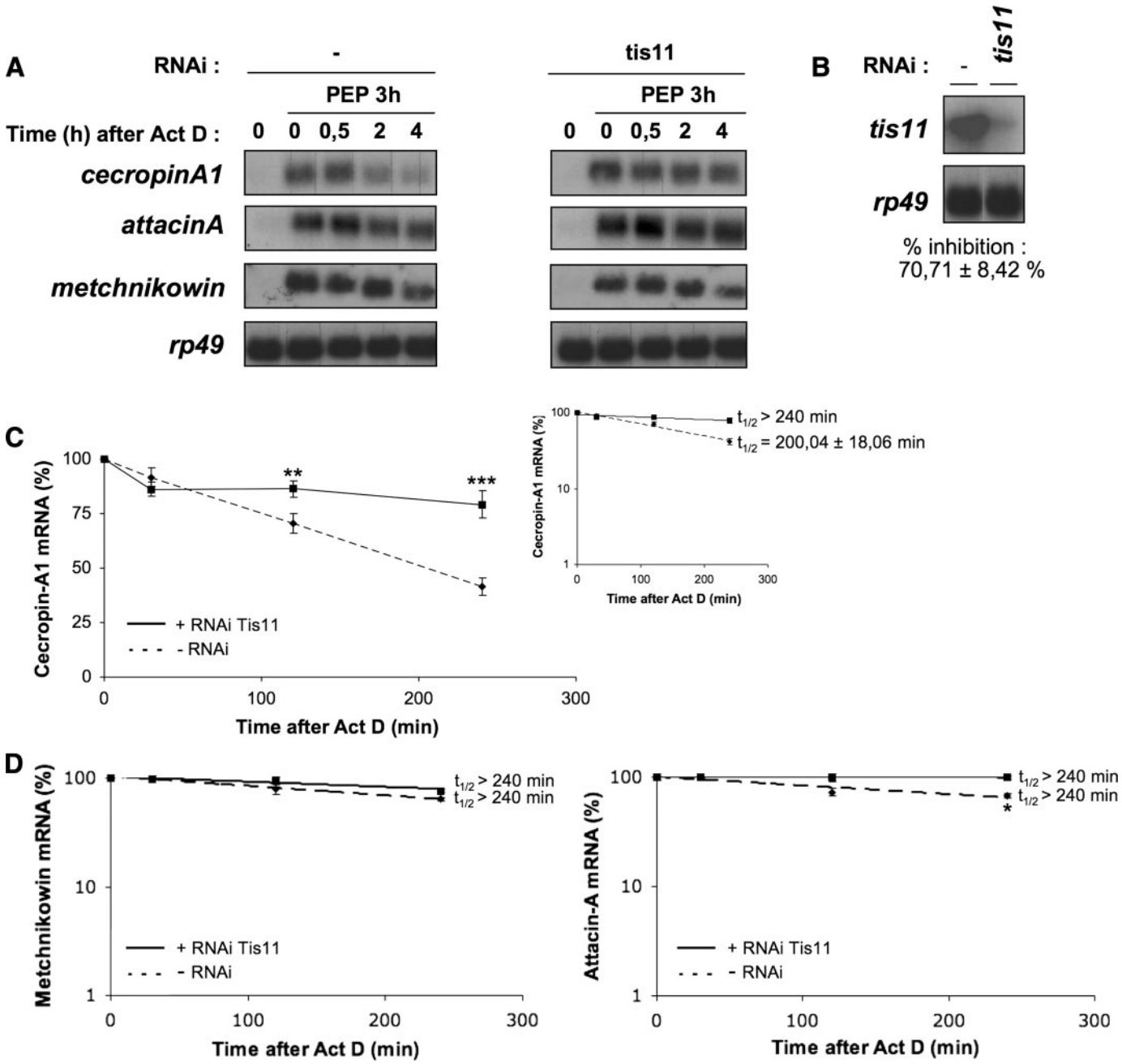

FIGURE 2. **Stability of AMPep mRNAs.** *A*, S2 cells were pretreated or not with double-stranded RNA directed against TIS11 for 3 days before induction with peptidoglycan (*PEP*) for 3 h and actinomycin D for the indicated times. Total RNA was extracted, and 8 μg were analyzed by Northern blot using the indicated riboprobes for hybridization. The results are representative of three independent experiments. *B*, *tis11* knock-down was quantified by northern blotting with a *tis11* antisense riboprobe. The inhibition efficiency was determined by quantification of three independent experiments. Equal loading was verified by detection of rp49 mRNA. *C*, quantification of CecA1 mRNA half-life in the presence and the absence of TIS11. *Main graph*, northern blots corresponding to seven independent experiments were quantified by PhosphorImager exposure or densitometric scanning. Results are expressed as the mean relative amount of CecA1 mRNA normalized on the intensity of the corresponding rp49 signal. The amount of mRNA at time 0 of the actinomycin D treatment was taken as 100% in each experiment and plotted on a linear scale graph. *Inset graph*, the same values were plotted on a semilogarithmic graph, and mRNA half-lives were determined by exponential regression. *D*, quantification of metchnikowin (*left panel*) and attacin A (*right panel*) mRNA half-lives in the presence or the absence of *tis11* dsRNA. Three independent northern blots performed as in *A* were quantified by PhosphorImager, values were plotted on a semilogarithmic graph, and mRNA half-lives were determined by exponential regression. *Error bars* represent S.D. for each time point. Mean values for samples corresponding to cells treated or untreated with *tis11* dsRNA were tested for statistical significance by two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

mutated to cytidines (supplemental Fig. 1*A*). As shown in Fig. 3*B*, the corresponding mRNA is stabilized as compared with the ARE-containing reporter mRNA ($t_{½} > 150$ min). This result demonstrates that the uridine residues flanking the AUUUA pentamers are necessary to the destabilizing activity of CecA1 ARE.

To confirm the influence of TIS11 on ARE-mediated mRNA decay, we performed experiments in which S2 cells were co-transfected with the DNA construct encoding GFP-TIS11 protein and the ARE or ΔARE reporter genes. As control, GFP alone was expressed in parallel. Northern blot analysis of reporter mRNAs after actinomycin D treatment revealed that

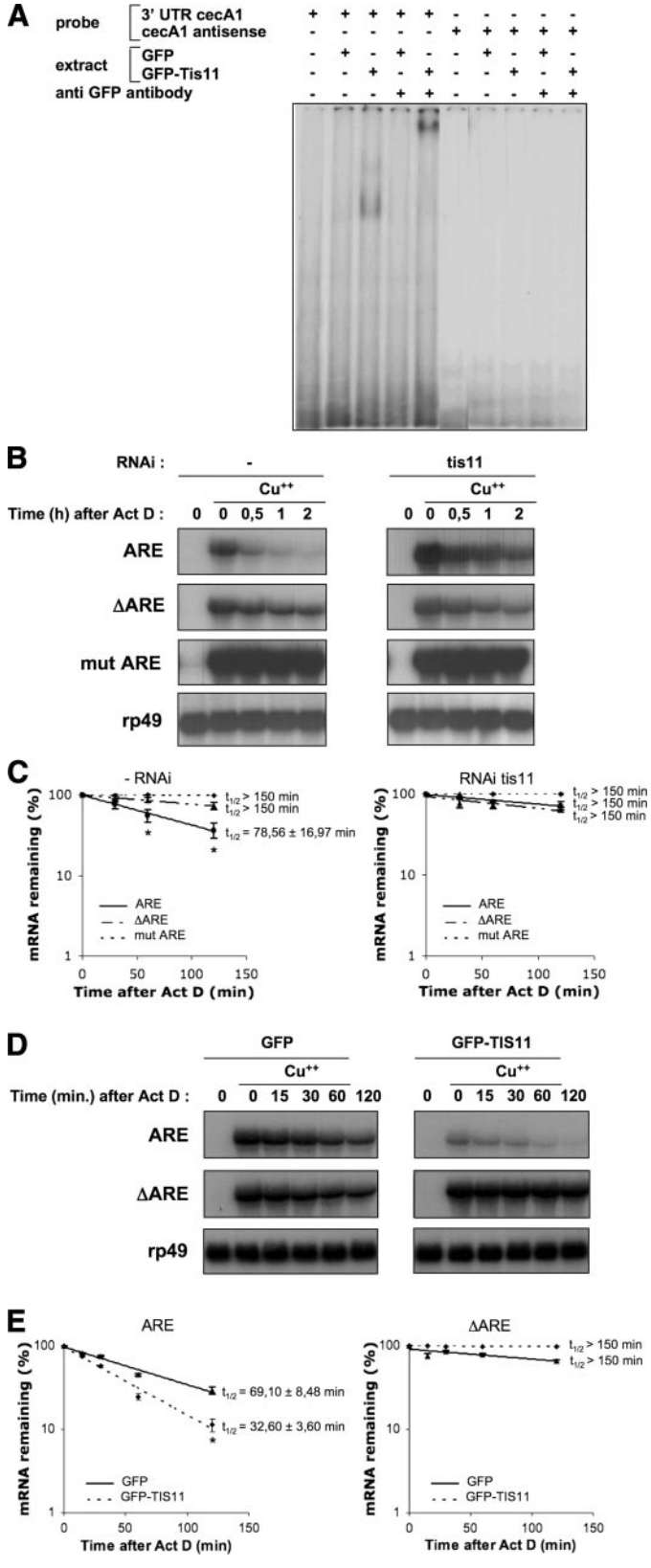

FIGURE 3. **TIS11 binds to CecA1 mRNA 3[prime]-UTR and destabilizes a reporter mRNA containing an ARE in the 3[prime]-UTR.** *A*, 10 μg of protein extract from cells transfected with the indicated expression vectors were incubated with ^{32}P-labeled probes in the presence or the absence of an anti-GFP antibody. Samples were treated with RNase T1 and heparin before loading on a 5% non-denaturing acrylamide gel. Migration was performed in Tris-buffered EDTA buffer. *B*, S2 cells were pretreated or not with *tis11* dsRNA, transfected with the indicated DNA construct, induced with $CuSO_4$, and treated with actinomycin D for the indicated times. Northern blot were hybridized with luciferase (*ARE*, *ΔARE*, *mut ARE*) and rp49 antisense riboprobes. *C*, quantification of reporter mRNA half-lives in the presence or the absence of *tis11* dsRNA. Three independent experiments performed as in *B* were quantified as described in the legend of Fig. 2, *C* and *D*. *D*, S2 cells were transfected with pMT-GFP or pMT-GFP-TIS11 expression vectors and pMT-Luc-CecA1 ARE or pMT-LucΔARE reporter genes, induced with $CuSO_4$ overnight, and treated with actinomycin D as noted. Reporter and rp49 mRNAs were detected as in *B*. *E*, quantification of reporter mRNA half-lives in the presence of GFP or GFP-TIS11 proteins. Three independent experiments performed as in *D* were quantified as described in the legend of Fig. 2, *C* and *D*.

the expression of GFP-TIS11 significantly decreased the stability of the ARE reporter mRNA as compared with its stability upon expression of GFP alone. In contrast, the ΔARE reporter mRNA stability was unaffected by GFP-TIS11 expression (Fig. 3, *D* and *E*). Altogether, these experiments demonstrate the existence of a functional ARE in CecA1 mRNA whose destabilizing activity strongly depends on TIS11 protein.

Imd Signaling Transiently Protects CecA1 mRNA from Degradation; Biphasic Deadenylation of CecA1 mRNA—The half-life of the reporter mRNA containing CecA1 full-length 3′-UTR ($t_{½}$ = 78 min, Fig. 3) in untreated S2 cells was inferior to the half-life of the endogenous CecA1 mRNA as measured in peptidoglycan-stimulated cells ($t_{½}$ = 200 min, Fig. 2). As Imd signaling is necessary to induce CecA1 transcription upon peptidoglycan exposure, we reasoned that this signal might transiently protect CecA1 mRNA from degradation. Therefore, we compared the degradation rate of CecA1 mRNA in S2 cells treated with peptidoglycan for 30 min or 3 h. As shown in Fig. 4*A*, CecA1 mRNA was unstable in cells activated for 3 h with peptidoglycan as more than 50% of the mRNA was lost after 3 h of actinomycin D treatment. In cells activated with peptidoglycan for 30 min, CecA1 mRNA was stabilized, as the total amount of CecA1 mRNA was virtually unchanged after 3 h of actinomycin D treatment and only decreased at later time points (5 h). In addition, this experiment confirmed that the size of CecA1 mRNA was higher at 30 min than at 3 h of peptidoglycan stimulation as previously observed (Fig. 1*A*), suggesting that CecA1 poly(A) tail length could be regulated by Imd signaling. We, therefore, evaluated CecA1 mRNA poly(A) tail after short and long exposures to peptidoglycan. This was performed by electrophoresis of RNAs on 2.5% agarose gel before northern blotting. The amount of total RNA was adjusted for each time point to obtain a CecA1 signal in the same detection range. The migration of CecA1 mRNA species was compared with *in vitro* deadenylated CecA1 mRNA obtained by RNase H degradation in the presence of oligo(dT). The longest form of CecA1 mRNA was observed after 30 min of peptidoglycan exposure (Fig. 4*B*, *lane 1*) and was significantly shortened upon further incubation (4 h) with actinomycin D (Fig. 4*B*, *lane 2*), suggesting that the mRNA is actively deadenylated. However, this size reduction is only partial as this mRNA species still migrated with a lower mobility than the *in vitro* deadenylated CecA1 mRNA species (Fig. 4*B*, *lane 5*). Of note, the size reduction observed after 4 h of transcriptional blockade was not modified by TIS11 inactivation (Fig. 4*B*, *lanes 3* and *4*), excluding any role of TIS11 in this early degradative process. After 3 h of peptidoglycan exposure, CecA1 mRNA was shorter

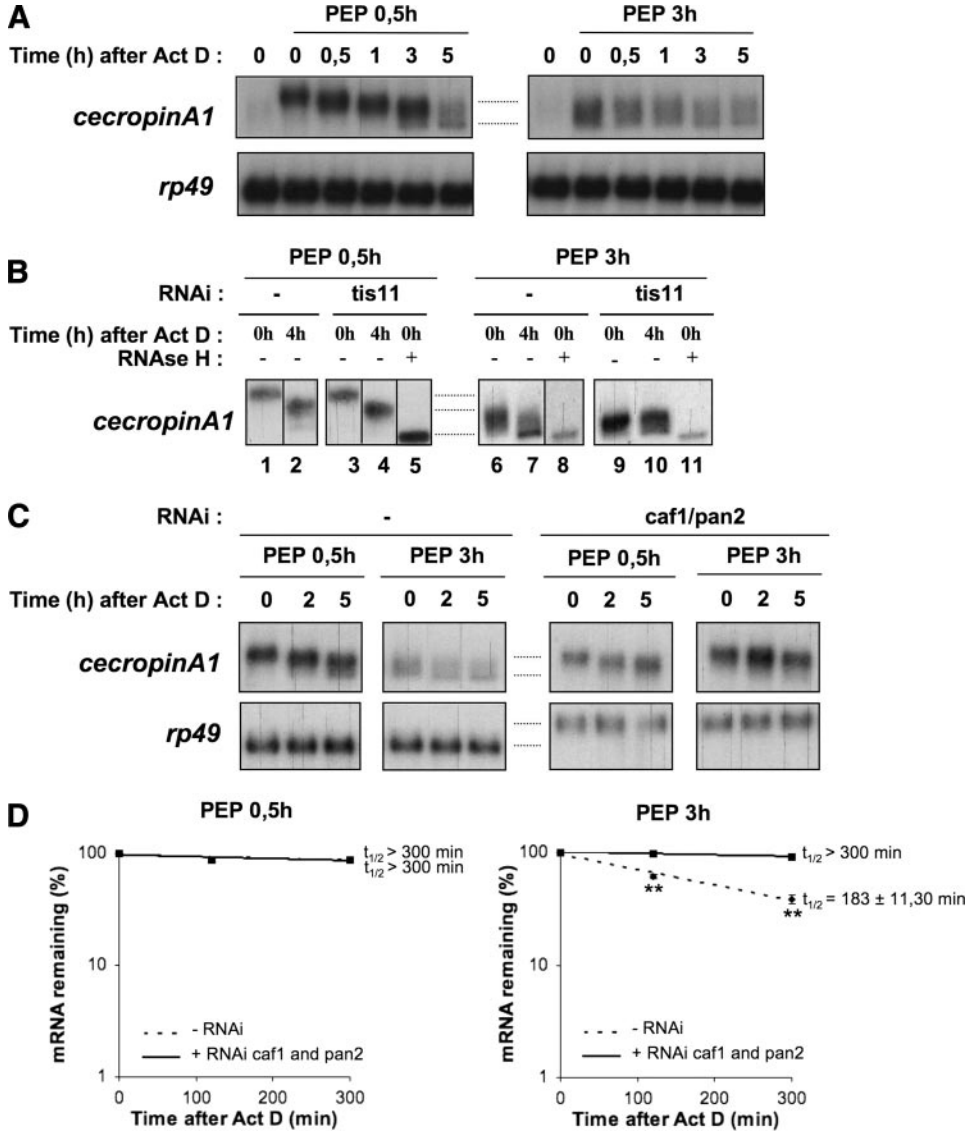

FIGURE 4. **Different stability and deadenylation of CecA1 mRNA after peptidoglycan (*PEP*) stimulation for 30 min or 3 h.** *A*, S2 cells were activated by peptidoglycan for 30 min or 3 h, and CecA1 mRNA degradation was analyzed as described in Fig. 2. *B*, RNA samples were treated or not with oligo(dT) + RNase H before analysis by Northern blot. The amount of total RNA was adjusted according to the intensity of the signals observed in *A* to detect similar CecA1 signals in every assay. The original image has been edited to show relevant samples. Samples not originally run side by side on the gel are separated by *vertical black lines* in the figure. *Dashed lines* indicate identical positions in separated parts of the original gel. *C*, S2 cells were pretreated with caf1 and pan2 dsRNAs. The stability of CecA1 mRNA was measured as in Fig. 2*A*. *D*, quantification of CecA1 mRNA half-life after 0.5 h (*left panel*) or 3 h (*right panel*) of stimulation with peptidoglycan in the presence (*continuous lines*) or the absence (*dotted lines*) of caf1 and pan2 dsRNAs. Each time point represents the mean ± S.D. of three experiments performed as in *C* and quantified as described in the legend of Fig. 2, *C* and *D*. Values were tested for statistical significance by two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

than after 30 min (Fig. 4*B*, compare *lane 1* and *6*) and migrated as a smear, suggesting that at this time point, CecA1 mRNA is heterogeneous in size. Subsequent incubation with actinomycin D (4 h) further reduced the mRNA size to that of the deadenylated form (compare *lanes 7* and *8*). Moreover, this deadenylation process was markedly reduced upon TIS11 inactivation (compare *lanes 7* and *10*).

In *Drosophila*, as in vertebrates, the major enzymatic complex involved in mRNA deadenylation is the CAF1·CCR4·NOT complex, although a minor contribution to the deadenylation process has also been assigned to the PAN2·PAN3 complex (28). To confirm that the size variation observed for CecA1 mRNA was related to a change in the poly(A) tail length, we measured CecA1 mRNA size after depletion of both CAF1 (product of pop2 gene) and PAN2 deadenylase activities by RNAi. As shown in Fig. 4, *C* and *D*, the inactivation of both enzymes induced a major increase in CecA1 mRNA length and stability at late time points (3 h) of peptidoglycan stimulation. The efficiency of CAF1 and PAN2 inactivation was confirmed by the size shift of RP49 mRNA.

We determined whether TIS11-dependent CecA1 mRNA deadenylation and degradation were under the control of CAF1 or PAN2 or both by inactivating them independently and assaying CecA1 mRNA size and decay. Results in Fig. 5, *A* and *B*, demonstrated that depletion of CAF1 only altered the TIS11-dependent late deadenylation and degradation process. Indeed, PAN2 depletion was without effect on CecA1 mRNA length and stability. CAF1 depletion did not modify CecA1 mRNA deadenylation after short times of peptidoglycan exposure (Fig. 5*D*, compare *lanes 2* and *5*). However, after 3 h of peptidoglycan stimulation, CAF1 depletion markedly reduced CecA1 mRNA deadenylation (Fig. 5*D*, compare *lanes 8–10*). Caf1 and pan2 gene expression was inhibited at similar levels (~60%) in these experiments (Fig. 5*C*).

To confirm that TIS11 and CAF1 depletions have the same effect on CecA1 mRNA deadenylation and to directly compare the size of samples previously shown on separated experiments, we compared the size of CecA1 mRNA obtained from S2 cells treated for 30 min or 3 h by peptidoglycan and actinomycin D (additional 4 h) in the presence or absence of caf1 or *tis11* dsRNA. As shown in Fig. 5*E*, CecA1 mRNA has a size of ~520 nucleotides after 30 min of peptidoglycan stimulation (*lane 1*). This size was reduced to ~450 nucleotides after 4 additional hours of actinomycin D treatment (*lane 2*). Treatment of the cells with peptidoglycan for 3 h and actinomycin D for 4 additional hours further reduced CecA1 mRNA size to ~350 nucleotides, corresponding to the size of the fully deadenylated molecule (compare *lanes 4* and *7*). However, depletion of *tis11* or caf1 blocked this late deadenylation phase, CecA1 mRNA being detected with a size of ~450 nucleotides (*lanes 5* and *6*). Alto-

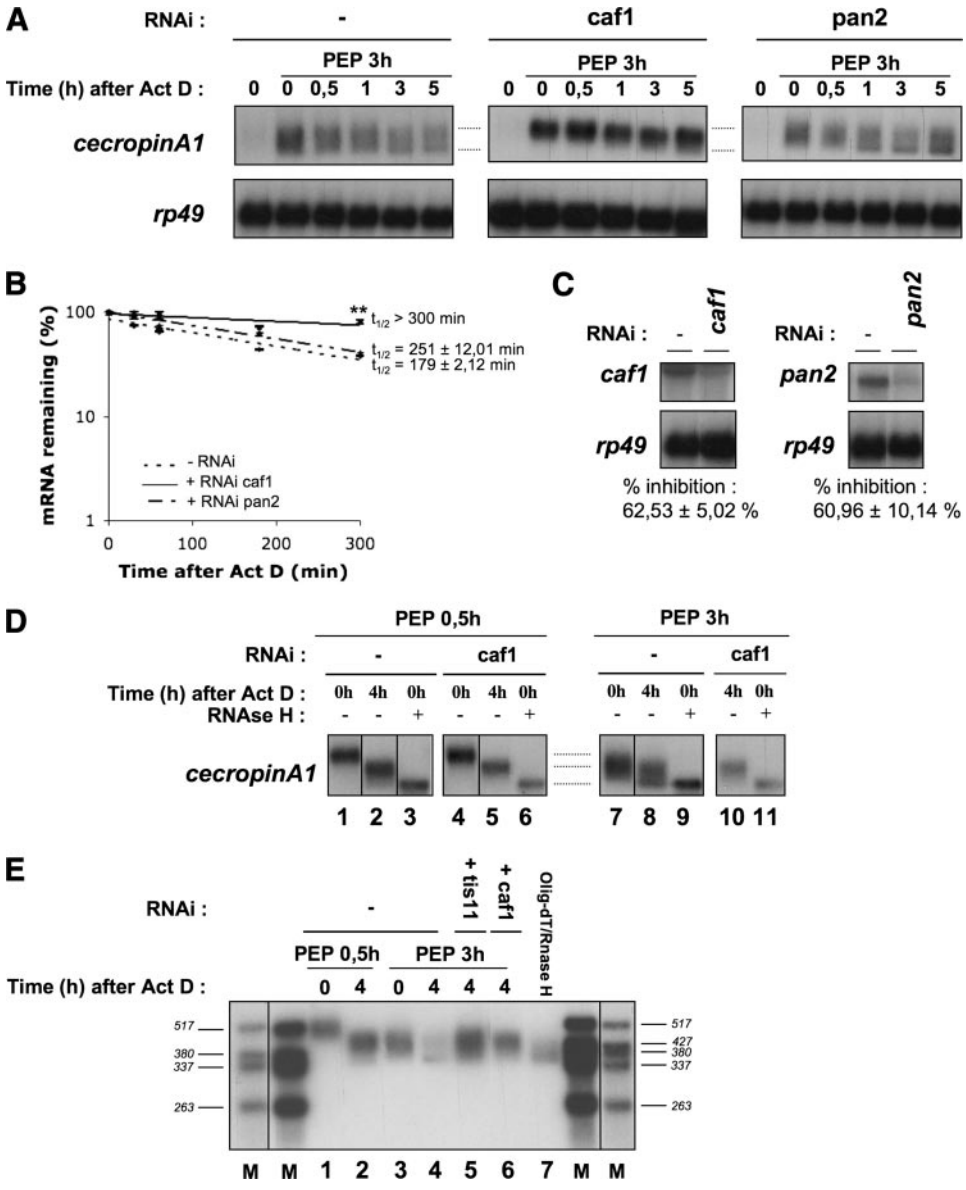

FIGURE 5. **Involvement of CAF1 in CecA1 mRNA deadenylation.** *A*, S2 cells were pretreated with caf1 or pan2 dsRNA, and the stability of CecA1 mRNA in cells stimulated with peptidoglycan (*PEP*) for 3 h was analyzed as described in Fig. 2*A*. *B*, quantification of CecA1 mRNA half-life in the absence or the presence of caf1 or pan2 dsRNA. Three independent experiments performed as in *A* were quantified as described in the legend of Fig. 2, *C* and *D*. *C*, knock-down of caf1 and pan2 by RNAi confirmed by Northern blot using caf1 and pan2 antisense riboprobes. The inhibition efficiency was determined by quantification of three independent experiments. Equal loading was verified by detection of rp49 mRNA. *D*, RNA samples were treated or not with oligo(dT) + RNase H before analysis by Northern blot. The amount of total RNA was adjusted according to the intensity of the signals observed in *A* to detect similar CecA1 signals in every assay. The original image has been edited to show relevant samples. Samples not originally run side by side on the gel are separated by *vertical black lines*. *Dashed lines* indicate identical positions in separated parts of the original gel. *E*, RNA samples from S2 cells treated as indicated were separated on the same gel with a RNA size marker, and CecA1 mRNA was detected by Northern blot. Sizes (*nt*) of the RNA marker are indicated.

gether, our data indicate that CecA1 mRNA is subject to a biphasic deadenylation process rapidly after its synthesis. Although the early phase is independent of TIS11, the later phase requires the expression of TIS11. Moreover, this later TIS11-dependent deadenylation phase depends on CAF1 deadenylase activity.

Up-regulation of tis11 Expression by Peptidoglycan Is Mediated by a Post-transcriptional Regulatory Mechanism—In light of the importance of TIS11 in the regulation of CecA1 mRNA, we explored *tis11* expression upon stimulation by peptidoglycan at the RNA and protein level. As shown in Fig. 6*A*, *tis11* mRNA production is similar in unstimulated and peptidoglycan-stimulated cells, indicating that *tis11* gene expression is not regulated at the transcriptional level by this stimulus. The two RNA species of ~2 kilobases as detected by Northern blot with *tis11* riboprobe most probably correspond to the previously described *tis11* mRNA isoforms (accession nos. NM_078586 and NM_167333). In contrast, we observed an increase in TIS11 protein accumulation upon peptidoglycan stimulation (Fig. 6*B*). Moreover, the basal level of TIS11 protein in unstimulated cells was up-regulated upon inhibition of the proteasome by clasto-lactacystine β-lactone. Of note, TIS11 migrated as two bands due to different phosphorylation status as verified by *in vitro* dephosphorylation with alkaline phosphatase (data not shown). To confirm that peptidoglycan-induced TIS11 accumulation does not rely on transcriptional activation, S2 cells were transfected with copper-inducible DNA constructs expressing GFP or GFP-TIS11. Cells were treated with copper sulfate for 1 h before peptidoglycan stimulation or were simultaneously incubated with copper sulfate and the proteasome inhibitor. As observed for TIS11 endogenous protein, GFP-TIS11 was significantly up-regulated by both peptidoglycan and the proteasome inhibitor and was detected as two bands, the slow migrating one corresponding to a phosphorylated form (not shown). In contrast, GFP and actin remained at the same levels in all conditions (Fig. 6, *B* and *C*). Altogether, these results demonstrate that *tis11* gene is constitutively transcribed in S2 cells. However, its accumulation is maintained at a low level by continuous degradation by the proteasome. Peptidoglycan stimulation leads to increased TIS11 accumulation most probably by reducing TIS11 protein turnover. This reduced TIS11 turnover might result from concomitant phosphorylation, preventing proteasome-mediated degradation. Consequently, ARE-containing mRNAs such as CecA1 undergo accelerated deadenylation and degradation after prolonged exposure to peptidoglycan.

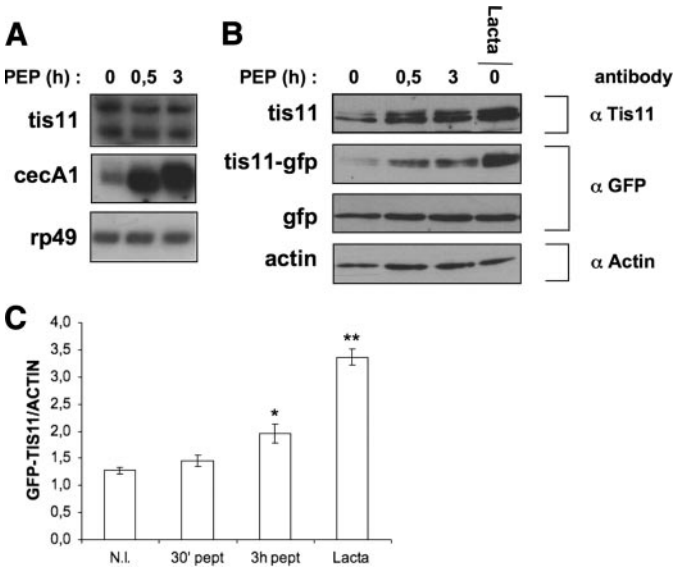

FIGURE 6. **Analysis of TIS11 gene expression in S2 cells.** *A*, analysis of TIS11 mRNA accumulation. S2 cells were treated with peptidoglycan (*PEP*) for the indicated times. Poly(A)$^+$ RNA was purified by chromatography on oligo(dT) agarose and analyzed by Northern blot using TIS11 antisense riboprobe. Equal loading of the samples was verified by detection of rp49 mRNA. The efficiency of S2 cells stimulation by peptidoglycan was confirmed by the induction of CecA1 mRNA. *B*, analysis of TIS11 protein levels by Western blot. Cells transfected with GFP- and GFP-TIS11-expressing plasmids were treated with peptidoglycan or with clasto-lactacystine β-lactone for the indicated times. Endogenous TIS11 was detected with a TIS11-specific antibody (see "Experimental Procedure"). GFP and GFP-TIS11 proteins were detected with a GFP-specific antibody. *C*, three independent experiments performed as in *B* were quantified, and the ratios of GFP-TIS11 and actin signals were plotted on the graph. *Error bars* represent S.D. for each experimental condition. Mean values were tested for statistical significance by two-tailed student's *t* test. *, $p < 0.05$; **, $p < 0.01$. N. I., non inducted.

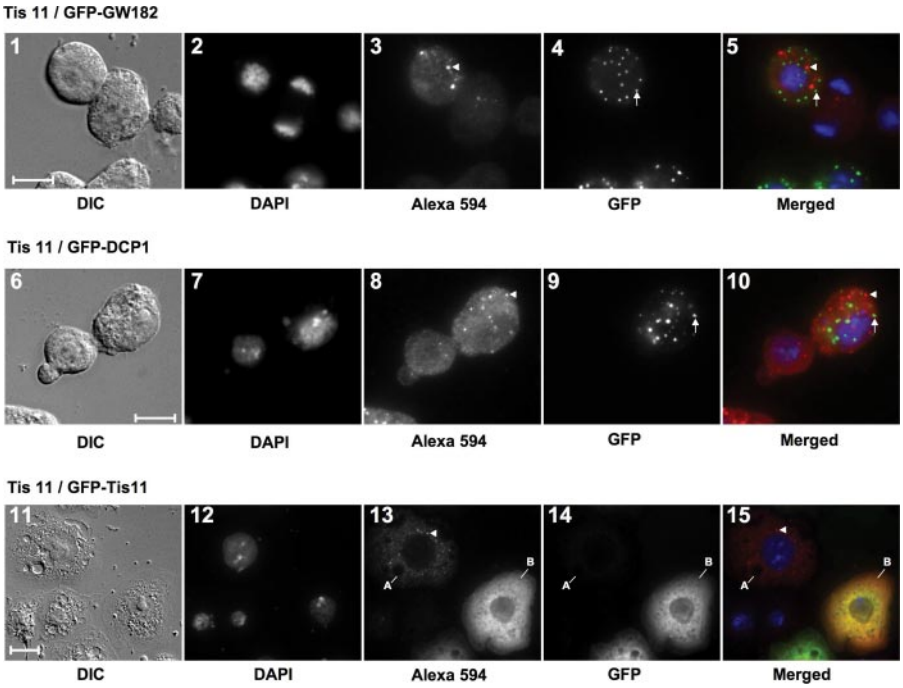

FIGURE 7. **Localization of TIS11, GFP-TIS11, GFP-GW182, and GFP-Dcp1 in S2 cells.** S2 cells were transfected with GFP-GW182 (*1–5*)-, GFP-Dcp1 (*6–10*)-, or GFP-TIS11 (*11–15*)-coding plasmids. Twenty-two hours after transfection cells were fixed and stained for TIS11 using a primary rabbit anti-TIS11 antibody (see "Experimental Procedures") and a secondary donkey anti-rabbit Alexa 594-coupled antibody (Molecular Probes) (*3*, *8*, and *13*), GFP-GW182 (*4*), GFP-Dcp1 (*9*), and GFP-TIS11 (*14*) localization was revealed by GFP direct fluorescence. Nuclear compartments were visualized by 4′,6-diamidino-2-phenylindole (*DAPI*) staining (*2*, *7*, and *12*). *Arrows* indicate P-bodies, and *arrowheads* indicate TIS11 foci. *Scale bar* = 10 μm. *DIC*, differential interference contrast.

Endogenous TIS11 Accumulates in Cytoplasmic Foci Distinct from Processing Bodies—Overexpression of TIS11 mammalian homologs TTP and BRF1 leads to their accumulation in Processing bodies (P-bodies), which are cytoplasmic structures specialized in RNA degradation. Under these conditions, TTP and BRF-1 were shown to address ARE-containing mRNAs to these structures (29, 30). We, thus, investigated TIS11 subcellular localization in S2 cells. S2 cells were transfected with GFP-TIS11, GFP-GW182, or GFP-Dcp1 constructs, and TIS11 intracellular distribution was revealed by immunofluorescence using anti-TIS11 antibody. We observed that endogenous TIS11 protein localized in discrete cytoplasmic foci (Fig. 7, *panels 3*, *8*, and *13*; see the *arrowheads*). In these cells P-bodies were identified by the localization of GFP-GW182 and GFP-Dcp1 proteins (Fig. 7, *panels 4* and *9*, respectively; see the *arrows*). Merged images of the 4′,6-diamidino-2-phenylindole, Alexa 594, and GFP fields revealed that TIS11 foci and P-bodies corresponded to distinct cytoplasmic structures (Fig. 7, *panels 5* and *10*). Unexpectedly, expression of DNA constructs encoding N-terminal GFP-tagged TIS11 led to a complete relocalization of TIS11 signal in the entire cytoplasmic compartment of transfected cells (Fig. 7, *panels 11–15*; compare endogenous TIS11 distribution in untransfected cell and GFP-TIS11 transfected cell). Of note, a similar relocalization of TIS11 signal was observed upon expression of a C-terminal FLAG-tagged TIS11 (not shown), thereby suggesting that this relocalization in the entire cytoplasm is, rather, due to TIS11 overexpression than to mislocalization induced by the GFP moiety. The GFP signal resulting from the expression of GFP-TIS11 perfectly co-localized with the signal detected by immunostaining with anti-TIS11 antibody (Fig. 7, *panels 13* and *14*, *bottom row*), thereby validating the specificity of TIS11 signal detected with anti-TIS11 antibody. Altogether, these results indicate that TIS11 accumulates in cytoplasmic foci distinct from P-bodies. However, overexpression of TIS11 leads to the disappearance of these TIS11-positive structures and relocalization of TIS11 in the entire cytoplasm. As the degradation of the ARE-containing reporter mRNA was enhanced by GFP-TIS11 expression (Fig. 3, *D* and *E*), we conclude that the accumulation of TIS11 in cytoplasmic foci is not necessary to induce the degradation of its mRNA targets.

DISCUSSION

Previous studies have demonstrated that the activation of the Imd pathway in S2 *Drosophila* cells leads to the induction of numerous genes classified in two groups based on different induction kinetics (9, 10). The first cluster gathers tran-

siently expressed genes under the transcriptional control of the JNK pathway, presenting a peak of induction approximately 2 h after stimulation followed by a rapid decline. This transient expression has been previously attributed to a rapid down-regulation of the JNK pathway resulting from the proteasomal degradation of TAK1 kinase. The second group includes genes having a sustained pattern of induction in response to peptidoglycan and which are controlled by the transcriptional activator Relish (10). In this work we observed that genes encoding several AMPeps display different patterns of induction in peptidoglycan-stimulated S2 cells. Indeed, *CecA1* and *attacin A* genes are transiently induced in response to peptidoglycan whereas the *metchnikowin* gene is sustainably activated in the same conditions. Moreover, RNAi experiments revealed that none of these genes are under the transcriptional control of the JNK pathway (Fig. 1).

Our data demonstrate that CecA1 mRNA is short-lived in S2 cells ($t_{1/2}$ = 200 min) and that depletion of the RNA-binding protein TIS11 by RNAi stabilizes this mRNA. We, thus, conclude that the transient pattern of expression of the CecA1 gene in response to peptidoglycan stimulation is mainly encountered by a powerful post-transcriptional down-regulation mediated by TIS11 protein.

Attacin A mRNA belongs to the group of transiently expressed genes. However, this mRNA is intrinsically more stable than CecA1 mRNA, and TIS11 depletion exerts a clear but moderate effect on its stability. These observations suggest that attacin A mRNA might be under the control of additional mechanisms remaining to be identified. Finally, TIS11 depletion had no effect on metchnikowin mRNA, thereby corroborating its sustained pattern of expression in response to peptidoglycan stimulation.

In mammals, proteins of the TIS11 family (TTP, BRF1, BRF2) mediate the destabilization of mRNA containing ARE in their 3′-UTR (12, 31). In *Drosophila*, previous studies report controversial evidence on the functionality of AU-rich sequences in this organism. Although Jing *et al.* (22) clearly showed that reporter genes containing AREs from mammalian origin are efficiently degraded in S2 *Drosophila* cells, another work (25) recently revealed that the AUUUA sequences present in the 3′-UTR of the *Drosophila* HSP70 mRNA are dispensable for the rapid deadenylation and decay of this mRNA. Our data indicate that the AU-rich sequence present in CecA1 mRNA acts a functional destabilizing ARE in *Drosophila* cells. We also show that TIS11 mediates CecA1 mRNA deadenylation and decay by direct binding to the 3′-UTR. Therefore, we conclude that *Drosophila* genes can be regulated at a post-transcriptional level by a cis-acting ARE via the recruitment of the trans-acting factor TIS11.

CecA1 mRNA 3′-UTR contains several repeats of the AUUUA pentamers in an overall AU-rich environment. In contrast, attacin A mRNAs contain only one AUUUA pentamer, and metchnikowin mRNA 3′-UTR bears an AU-rich region but devoid of AUUUA motifs (see supplemental Fig. 1). In mammals, class I AREs were defined as sequences containing 1–3 repeats of the AUUUA pentamer scattered in the 3′-UTR, whereas class II AREs should contain overlapping pentamers and/or AUUUA pentamers directly flanked by U residues (such as (UU)AUUUA(UU)) (26, 27, 32). Here, we showed that the mutation of U residues flanking AUUUA motifs in the CecA1 mRNA 3′-UTR abolished the ARE-destabilizing effect, demonstrating that several copies of the AUUUA pentamers scattered in the 3′-UTR of the mRNA are not sufficient to form a functional ARE in *Drosophila*. Interestingly, the AUUUA pentamer present in attacin A mRNA is flanked by one U residue (supplemental Fig. 1), thereby allowing this motif to contribute to the TIS11-dependent degradation of the mRNA. Altogether, these and our observations suggest that class II ARE have been evolutionary conserved between invertebrates and mammals. On the contrary, class I AREs appear not to be active in *Drosophila* cells and, therefore, probably arose later in evolution as mRNA-destabilizing elements.

CecA1 mRNA is deadenylated by a biphasic mechanism of degradation after induction by peptidoglycans. Interestingly, TIS11, although dispensable in the early deadenylation phase, is required for the later step. Several reports indicate that TIS11 mammalian homologs TTP and BRF1 induce the degradation of target mRNAs by promoting their deadenylation. However, the mechanisms underlying this process are not fully understood (21, 33, 34). We investigated the influence of deadenylase complex subunits on CecA1 mRNA degradation and decay. Our data show that the late TIS11-dependent deadenylation phase of CecA1 mRNA requires a CAF1 complex, thereby suggesting that TIS11-dependent deadenylation and decay are exerted via the CCR4·NOT·CAF1 complex in *Drosophila*. The mechanism responsible for TIS11-dependent recruitment of this complex on target mRNAs has not been investigated so far. It should be noted, however, that the N-terminal region of TTP, which has been shown to recruit CAF1·CCR4·NOT, shares a low degree of similarity with *Drosophila* TIS11 protein (21). Therefore, it would be of interest to determine whether TIS11 interacts with the deadenylation complex by a mechanism similar to what is observed in mammals or if other factors are involved.

In mammalian cells the expression of genes belonging to *tis11* family is highly regulated at the transcriptional level by different signaling pathways (35, 36). TTP and BRF-1 levels are also regulated by a post-translational mechanism in which the phosphorylation status of these proteins controls their degradation by the proteasome (37–39). We observed that, unlike its mammalian counterparts, the *Drosophila tis11* gene is regulated by the Imd pathway solely by a post-transcriptional mechanism. Indeed, TIS11 mRNA is equally expressed in unstimulated and peptidoglycan-stimulated cells. However, TIS11 accumulation is maintained at low levels in unstimulated cells and is up-regulated upon peptidoglycan stimulation most probably by a stabilization process depending on TIS11 protein phosphorylation. The existence of a common pathway (*e.g.* Imd) regulating the transcriptional activation and post-transcriptional down-regulation of CecA1 mRNA contributes to the establishment of a coordinated regulatory network ensuring a transient expression of this gene. These mechanisms appear to have become more complex later in evolution as testified by the emergence of a transcriptional control on the synthesis of the post-transcriptional regulator in vertebrates.

We observed that endogenous *Drosophila* TIS11 protein accumulates in cytoplasmic granules distinct from P-bodies. However, we show that ectopic expression of TIS11 induces the relocalization of TIS11 from these structures to the entire cytoplasm without affecting the efficiency of TIS11-mediated mRNA degradation. We conclude that TIS11 localization in discrete cytoplasmic foci is not required for its activity in *Drosophila* cells, and therefore, the function of these TIS11 granules remains to be investigated. These structures could constitute reservoirs of TIS11 protein and may not be sites of effective mRNA degradation. Determination of the subcellular localization of CecA1 mRNA and of deadenylation complexes in S2 cells could help clarify this point.

The ARE-mediated mRNA destabilizing mechanism identified here is probably one among several mechanisms involved in the down-regulation of genes activated by the Imd and Toll pathways (40). The variety of these processes underlines the importance for the fly to temper the expression of genes activated in response to infectious agents and in particular AMPep genes. The reasons why such strong negative controls are needed is not completely understood. In mammals, down-regulation of inflammatory cytokine production by several mechanisms including ARE-mediated post-transcriptional control limits cytokine toxicity toward the host. Several arguments suggest that the protection of host cells might not be the major reason for down-regulating AMPep production in *Drosophila*. AMPeps can be secreted at very high concentrations (up to the mM range) without apparent toxicity for the host (41). The constitutive expression of certain AMPeps including CecA in several fly organs (2) also argues against a strong toxicity of these molecules toward the host. Recently, Ryu *et al.* (42) demonstrated that the homeobox gene *caudal* contributes to the maintenance of the commensal flore in the gut by negatively regulating the expression of AMPep genes. We can, therefore, speculate that the panoply of negative controls regulating AMPep gene expression including ARE-mediated post-transcriptional control, might be of importance to reach an equilibrium between the efficient clearance of pathogenic microorganisms and the preservation of microorganisms beneficial to the host.

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