A humoral stress response in *Drosophila*

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The ability to react to unfavorable environmental changes is crucial for survival and reproduction, and several adaptive responses to stress have been conserved during evolution [1–3]. Specific immune and heat shock responses mediate the elimination of invading pathogens and of damaged proteins or cells [4–6]. Furthermore, MAP kinases and other signaling factors mediate cellular responses to a very broad range of environmental insults [7–9]. Here we describe a novel systemic response to stress in *Drosophila*. The *Turandot A* (*TotA*) gene encodes a humoral factor, which is secreted from the fatbody and accumulates in the body fluids. *TotA* is strongly induced upon bacterial challenge, as well as by other types of stress such as high temperature, mechanical pressure, dehydration, UV irradiation, and oxidative agents. It is also upregulated during metamorphosis and at high age. Strikingly, flies that overexpress *TotA* show prolonged survival and retain normal activity at otherwise lethal temperatures. Although *TotA* is only induced by severe stress, it responds to a much wider range of stimuli than heat shock genes such as *hsp70* or immune genes such as *Cecropin A1*.

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Results and discussion

A bacterially induced humoral factor

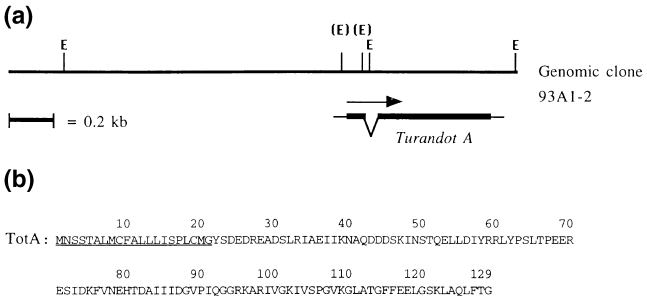
We initially identified the *Turandot A* (*TotA*) gene in a screen for bacterially induced genes by comparing infected and unchallenged flies by differential display [10]. Sequence analysis of a full-length cDNA clone predicts a protein of 14 kDa with an N-terminal signal sequence and suggests that TotA is exported as a 12 kDa peptide. The mature protein is acidic and highly charged and does not contain cysteine (Figure 1b). It is not obviously related to any previously described protein, and its existence was not predicted by the *Drosophila* genome project. However, a database search indicates that a small family of related open reading frames is present in the *Drosophila* genome (data not shown).

We have also isolated genomic clones covering the *TotA* gene. The sequence of these clones, as well as that determined by the *Drosophila* genome project, reveals that *TotA* has two exons interspaced by an 86 nucleotide-long intron (Figure 1a). In situ hybridization to chromosomal squashes showed that *TotA* is localized at 93A1–2 on the right arm of the third chromosome; these results are in good agreement with data from the genome project.

As expected from the differential display screen, *TotA* is strongly induced after a bacterial challenge. The injection of live bacteria into adult flies gives a 10- to 20-fold increase in the transcriptional activity (Figure 2a, compare lanes 7 and 8). During development, *TotA* is also expressed at a low level in unchallenged animals (Figure 2a, lanes 1–6). The 0.7 kb transcript first appears in late larval stages and remains expressed during metamorphosis. This constitutive expression ceases as soon as the fly is ready to eclose. Most young flies express little or no *TotA*, but there is considerable individual variation and about 5% of all flies express *TotA* without any intentional challenge (not shown). Starting about 3–4 weeks after eclosure, there is a gradual increase of *TotA* transcription in adult flies (Figure 2b).

We localized the major site of *TotA* transcription to the fatbody (Figure 2c), an organ known to express many hemolymph proteins. Western blots of similarly dissected material confirm that the TotA protein is present in the fatbody, both in bacterially challenged and unchallenged third-instar larvae, whereas the remaining tissues are essentially devoid of this protein (Figure 2d). The estimated size of the detected protein is approximately 12 kDa, in good agreement with the expected value. TotA protein was also found in the hemolymph, and this result confirms

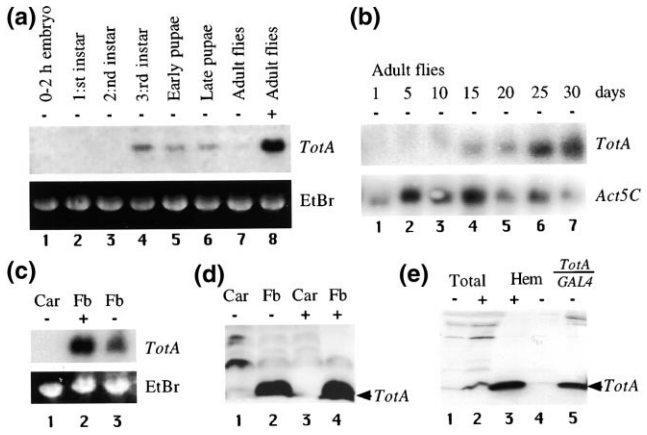
Figure 1



The *TotA* gene and its product. **(a)** The genomic organization of the *TotA* gene. EcoRI restriction sites are labeled "E." The restriction sites in parenthesis are missing in some genomic clones due to allelic variation. Coding parts of the *TotA* transcript are shown as thick lines. **(b)** Amino acid sequence of TotA. The predicted signal sequence is underlined.

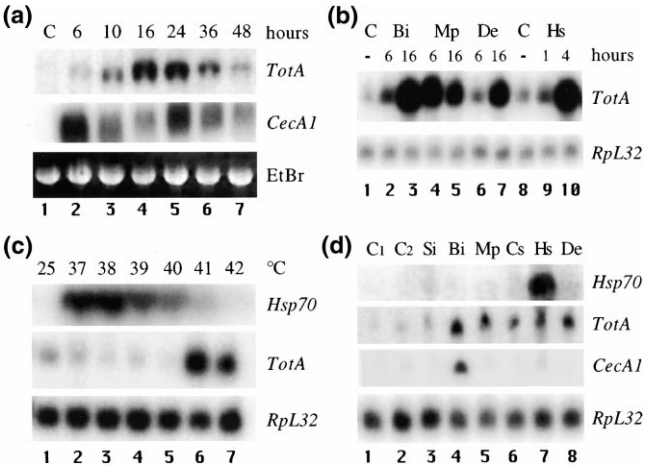
that the protein is secreted (Figure 2e). The increase in the TotA level after bacterial induction is more pronounced in hemolymph (compare lanes 4 and 3) than in total extract (compare lanes 1 and 2), and this finding

Figure 2



Expression of *TotA* in untreated animals (-) or animals injected with bacteria (+). **(a)** A Northern blot with RNA extracts from different developmental stages was probed with ^{32}P-labeled *TotA* cDNA. The ethidium bromide-stained gel is shown as a loading control. **(b)** Age-dependent expression. RNA from unchallenged adult flies of increasing age was blotted and probed with ^{32}P-labeled *TotA* cDNA. The blot was reprobed with *Act5C* as a control. **(c)** Fatbody localization of *TotA* transcription is shown by a Northern blot of RNA from the fatbody (Fb) of third-instar larvae. Lane 1 contains RNA from the remaining carcass (Car). As a control for loading, we show the ethidium bromide-stained gel. **(d)** Fatbody localization of TotA protein is assayed by a Western blot. Protein extracts from the fatbody or the remaining carcass of third-instar larvae. There are 50 μg total protein per lane. **(e)** Western blot of total extracts (Total) of adult flies, with 1.5–2 animals per lane, or of hemolymph (Hem), from 13 animals per lane. Lane 5 contains hemolymph from 13 uninjected *TotA*-overexpressing flies.

Figure 3



Stress induction of *TotA*. **(a)** Bacterial induction of *TotA* and *CecA1*. RNA was extracted from adult flies at the indicated time points after injection with *E. cloacae* β*12* and analyzed on a Northern blot probed with *TotA* and *CecA1*, respectively. "C" indicates the uninjected control. As a control for loading, we show the ethidium bromide-stained gel. **(b)** Stress induction, assayed by Northern blot. RNA was extracted from adult flies 6 or 16 hr after treatment. Heat-shocked flies were taken immediately after 1 or 4 hr of heat exposure (37°C). "C" indicates the untreated control; "Bi" indicates bacterial injection; "Mp" indicates mechanical pressure; "De" indicates dehydration; and "Hs" indicates heat shock. The filter was reprobed with *RpL32* as loading control. **(c)** Heat induction. RNA extracts from control flies (25°C) and flies kept for 22 min at the indicated higher temperatures (lane 2–7) were assayed by Northern blot, probed with *Hsp70*, *TotA*, and *RpL32*-cDNA, respectively. **(d)** Stress induction. RNA samples were extracted from flies 12 hr after each treatment. "C1" indicates untreated flies; "C2" indicates flies anaesthetized with CO_2; "Si" indicates sterile injection; "Bi" indicates bacterial injection; "Mp" indicates mechanical pressure; "Cs" indicates cold shock (4°C for 4 hr); "Hs" indicates heat shock (37°C for 4 hr); and "De" indicates dehydration. The filter was probed with *Hsp70*, *TotA*, *CecA1*, and *RpL32* cDNA, respectively.

indicates that the secretion of TotA may also be a regulated process.

TotA is induced by stress as well as bacterial challenge

Although *TotA* is induced by bacteria, this gene differs in several respects from immune response genes such as *Cecropin A1* (*CecA1*). For instance, the kinetics of induction is entirely different. Whereas *CecA1* is induced immediately after a bacterial injection, *TotA* is activated gradually and reaches a peak after 16 hr (Figure 3a). Moreover, baculovirus-expressed TotA protein does not show any antimicrobial activity, and TotA-overexpressing flies do not show increased resistance to infection compared to wild-type flies. In addition, mutants in the immune response pathways have no clear effect on *TotA* induction (data not shown). Tested mutants include the *Toll* gain-of-function mutant as well as *Toll*, *imd*, and *Relish* loss-of-function mutants. Thus, TotA does not appear to be part of the immune response.

Considering the fact that *TotA* is also activated during metamorphosis and in aging flies, we thought that the slow onset of its expression in infected flies might instead reflect a late response caused by the stress of infection. Metamorphosis is a potentially stressful period of intense cellular turnover, and the increased constitutive expression of *TotA* with age could be due to the fact that old cells are more sensitive to environmental stress [11]. Therefore, we proceeded to investigate if other stress-related stimuli also activate *TotA* transcription. We found that *TotA* is strongly induced by mechanical pressure, dehydration, and heat (Figure 3b). Other well-known stress response inducers such as UV-irradiation and the oxidative reagent paraquat also act as strong gene activators (data not shown), and these findings confirm the broad activation spectrum of *TotA*.

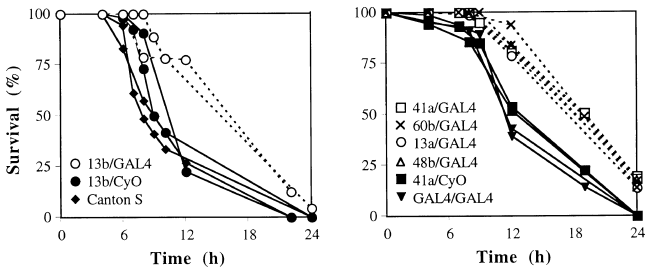
We next compared the heat activation of *TotA* with that of a known heat shock response gene, *Hsp70*. Although both are heat inducible, Figure 3c shows a clear difference in the temperature dependence of gene activity. *Hsp70* is strongly activated after a short exposure to moderate heat, with a maximum around 38°C, while *TotA* is acutely expressed only when the flies are subjected to the severe stress of very high temperatures above 40°C. However, *TotA* can also be induced at 37°C, but only after prolonged exposure (Figure 3b).

When we compare the induction of *TotA* by different stimuli to that of the heat shock gene *Hsp70* and the immune response gene *CecA1*, the difference is obvious. *Hsp70* is primarily induced by heat, and *CecA1* by bacteria. In contrast, *TotA* transcription is activated by both treatments as well as by several other stress factors (Figure 3d). Hence, *TotA* responds to a broader range of stimuli than the heat shock and immune genes.

Flies overexpressing *TotA* are more resistant to heat stress

To further analyze the function of the TotA protein, we generated transgenic flies that express *TotA* under control of the GAL4-UAS system [12]. Five independent transgenic strains were isolated and crossed to c729, a constitutive GAL4 driver that is expressed in several tissues, including fatbody and lymph glands [13]. All five strains correctly expressed the transgene without any previous challenge, as monitored by Northern and Western blots. An example of TotA protein overexpression is shown in Figure 2e (compare lanes 5 and 4). Although the expression of TotA under this driver has no obvious effect on the flies at normal temperature, it has a strong effect on their response to heat stress. Whereas wild-type flies become very slow and sluggish after a few hours at 37°C, the TotA-overexpressing flies remain active and motile. These flies also survive several hours longer at otherwise

Figure 4



Increased heat resistance of *TotA*-overexpressing flies. *TotA* is overexpressed in the transgenic stocks 13a, 13b, 41a, 48b, and 60b when crossed to the GAL4 driver c729 (dashed lines), but not in the controls that carry the balancer chromosome CyO, the homozygous GAL4 driver only, or in wild-type Canton S flies (solid lines). The diagrams show the percent surviving flies after different times of incubation at 37°C. Each panel shows a separate set of experiments, with two repetitions for each stock except 13a/GAL4 and 48b/GAL4. Between 120 and 300 female flies were used for each survival curve.

lethal conditions. Figure 4 shows two series of experiments with the five independent transgenic *TotA* strains, crossed to the c729 driver. As compared to their driverless siblings, the homozygous c729 driver strain alone, or a wild-type Canton S strain, the progeny showed prolonged survival when they were kept at 37°C. Compared to that of the four controls, the median survival time for the five stocks that overexpress *TotA* was significantly prolonged by about 7 hr ($p < 0.0005$, Student's t test).

With the c729 driver, the *TotA*-overexpressing flies mature normally, and in the absence of stress they show no obvious morphological phenotype either during development or as adults. However, *TotA* overexpression is lethal when driven by *P{Act5C-GAL4}25FO1*, a strong constitutive driver with an early onset and ubiquitous expression. When crossed to this driver and raised at 25°C, the progeny of all five transgenic strains die during the early larval stages, whereas a few survivors are seen when reared at 18°C (data not shown).

The protection against heat stress provided by *TotA* overexpression is quite impressive, considering that even normal flies produce high levels of this protein when they are stressed. It remains to find out how TotA exerts this protective effect. It is possible that it acts as an extracellular counterpart to the heat shock proteins by dealing with denatured proteins in circulation or on cell surfaces. TotA could also be involved in tissue repair, or it could perhaps serve as a stress hormone and affect the physiology of the stressed fly. However, *TotA* overexpression does not lead to induction of the heat shock, immune, or endogenous *TotA* genes. This also indicates that the overexpression of TotA does not in itself act as a stress factor that activates unspecific protective responses in the organism.

The induction of TotA represents a novel type of stress response. Like many of the heat shock proteins, it is induced by a broad range of environmental stresses. However, unlike the heat shock proteins, TotA is a humoral factor with systemic effects. Furthermore, the induction of *TotA* is only partially correlated with that of the heat shock genes, and this finding indicates that the two responses are most likely controlled by different mechanisms. Consistent with this conclusion, we found no obvious consensus binding site for the heat shock factor in the *TotA* promoter region. As discussed above, *TotA* induction is probably also mechanistically unrelated to the immune response. There are other distinct stress-related signaling pathways, involving molecules such as octopamine, STAT and p38 [3, 14, 15]. Preliminary experiments on *TotA* induction in mutants that affect these different pathways have so far not been informative. However, as TotA is activated by a broad variety of inducers, there is a fair chance that multiple signaling pathways converge on *TotA* activation. To fully rule out the contribution of one specific pathway would require multiple simultaneous mutations in several different pathways.

The discovery of this new type of response in *Drosophila* is an example of a systemic adaptation of the organism to acute stress. Turandot A does not only protect against immediate death but also makes the flies considerably more active during periods of heat stress. Such broad systemic effects should be beneficial for survival in a natural environment.

Materials and methods

Flies, stress treatment

Wild-type Canton S flies were used for most experiments. The GAL4 driver stocks c729 and *P{Act5C-GAL4}25FO1* were obtained from the Bloomington *Drosophila* Stock Center, Indiana University. All flies were reared at 25°C on standard yeast/agar media. Unless otherwise indicated, 2- to 3-day-old adults were used in the experiments.

For infection, we dipped the animals into 85% ethanol before injecting approximately 0.15 μl of an overnight culture of *Enterobacter cloacae* β12, diluted 1:10 in *Drosophila* Ringer (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris-HCl, [pH 7.2]). Sterile injections were performed with Ringer only. Surviving animals were frozen in liquid nitrogen. Unless otherwise indicated, larvae were taken 6 hr after injection, and adults were taken 16 hr after injection. We used untreated animals as a control.

Other stress induction experiments were performed as follows: For dehydration, flies were placed on silica gel for 90 min. We applied mechanical pressure by squeezing the thorax with a pair of forceps without rupturing the cuticle. The flies were then kept under standard conditions for 6 or 16 hr before freezing. Flies were heat shocked or cold shocked in glass vials with a moist filter paper. These were immersed into a thermostated water bath for the indicated time. Immobile flies were discarded before freezing.

Developmental stage, dissection

To collect synchronized embryos, we allowed females to lay eggs for 5 hr. These eggs were aged at 25°C on yeast/agar media, isolated, and quick-frozen at the different developmental stages assayed.

For dissection, third-instar larvae were washed in Ringer. The fatbody and remaining carcasses were separately quick-frozen in liquid nitrogen. Each preparation was split into two parts used for RNA and protein extraction, respectively. Hemolymph from adult flies was collected in thin glass capillaries, diluted with Ringer, and centrifuged for 2 min at 2000 g before the supernatant was frozen.

Generation of transgenic flies

The 669 bp NotI-KpnI insert of the *TotA* cDNA clone was inserted between the corresponding sites in the pP{UAST} vector [12]. The construct, pP{UAS-TotA}, was used for the generation of flies transgenic for TotA under the UAS promoter by P-element mediated transformation [16]. By this technique, we generated five independent fly strains, Tot13a, 13b, 41a, 48b, and 60b, with insertions of the transgene *P{UAS-TotA}* in different positions on the chromosomes X and 2.

Survival experiment

To assay heat resistance, we crossed virgin females of P{UAS-TotA}13a/P{UAS-TotA}13a, P{UAS-TotA}13b/CyO, P{UAS-TotA}41a/CyO, P{UAS-TotA}48c/P{UAS-TotA}48c, or P{UAS-TotA}60b/P{UAS-TotA}60b to c729 males that carry the GAL4-producing insertion P{GawB}c729 [13]. Resulting P{UAS-TotA}/P{GawB}c729 females, at 1–4 days of age, were kept at 37°C in glass vials with moist filter paper. Survival was checked regularly. The strains used for control (Canton S, c729, P{UAS-TotA}13b/CyO and P{UAS-TotA}41a/CyO) were identically treated.

Supplementary material

Supplementary material with additional methodological details is available at http://images.cellpress.com/supmat/supmatin.htm.

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