α-actinin accounts for the bioactivity of actin preparations in inducing STAT target genes in Drosophila melanogaster

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Abstract

Damage-associated molecular patterns (DAMPs) are molecules exposed or released by dead cells that trigger or modulate immunity and tissue repair. In vertebrates, the cytoskeletal component F-actin is a DAMP specifically recognised by DNGR-1, an innate immune receptor. Previously we suggested that actin is also a DAMP in Drosophila melanogaster by inducing STAT-dependent (Srinivasan et al., 2016). Here, we revise that conclusion and report that α-actinin is far more potent than actin at inducing the same STAT response and can be found in trace amounts in actin preparations. Recombinant expression of actin or α-actinin in bacteria demonstrated that only α-actinin could drive the expression of STAT target genes in Drosophila. The response to injected α-actinin required the same signalling cascade that we had identified in our previous work using actin preparations. Taken together, these data indicate that α-actinin rather than actin drives STAT activation when injected into Drosophila.
Introduction

Metazoan organisms need to be able to recognise damaged tissues in order to induce processes that promote tissue repair and that prevent infection caused by barrier breach. Tissue damage is generally accompanied by cell death and release of damage-associated molecular patterns (DAMPs), molecules that are sequestered within healthy cells but become exposed to the extracellular milieu upon loss of membrane integrity and that trigger inflammation, modulate immunity or promote tissue repair (Rock, Latz, Ontiveros and Kono, 2010; Zelenay and Reis e Sousa, 2013). Examples of DAMPs include ATP, uric acid, IL-33, IL1α, RNA and DNA (Zitvogel, Kepp and Kroemer, 2010), as well as actin, a ubiquitous and abundant component of the cytoskeleton of all eukaryotes. Exposure of filamentous (F-) actin by dead cells can be recognised by an innate immune receptor known as DNGR-1 (aka CLEC9A) expressed by specialised leucocytes (Ahrens et al., 2012; Hanč et al., 2015; Zhang et al., 2012). DNGR-1 is only found in mammals but we recently provided evidence that cytoskeletal exposure may be sign of cell damage also in Drosophila melanogaster. In Drosophila, mechanical and other stresses elicit JAK-STAT pathway signalling, which is implicated in stem cell mobilisation and tissue repair (Ekengren and Hultmark, 2001; Ekengren et al., 2001; Agaisse et al., 2003; Brun et al., 2006; Jiang et al., 2009). We found that injection of purified actin into adult flies led to selective induction of STAT target genes in the fat body, the Drosophila equivalent of the mammalian liver (Srinivasan et al., 2016). The response to actin required the NADPH oxidase Nox, the Src family kinase Src42A, and the adapter Shark leading to an autocrine and paracrine amplification loop that involved the cytokine Upd3 and the JAK-STAT-coupled cytokine receptor Domeless (Srinivasan et al., 2016). Here, we revise our interpretation of those data and report that alpha-actinin (α-actinin), a
cytoskeletal protein tightly associated with F-actin, is a more potent inducer of STAT target genes than actin. Like the response to actin, the response to α-actinin requires Nox, Src42A and Shark. Notably, α-actinin can be found in trace amounts in the purified actin preparations that we had used in our initial study and recombinant actin expressed in bacteria and devoid of α-actinin is no longer capable of eliciting the STAT response upon injection into flies. We conclude that α-actinin rather than actin is the key trigger of STAT activation upon injection into Drosophila, suggesting that distinct cytoskeletal proteins can serve as DAMPs across species.

Results and Discussion

In our previous study, actin purified from human platelets or rabbit muscle or recombinant actin purified from insect cells elicited the expression of STAT-responsive genes in the fat body when injected into adult Drosophila melanogaster (Srinivasan et al., 2016). While assessing the ability of other cytoskeletal proteins to elicit a similar response, we found that myosin, α-actinin, and, to a lesser degree, tubulin could also drive induction of the STAT responsive gene TotM (Figure 1a). On a per molecule basis, α-actinin was the most potent trigger and was superior to myosin, the second most potent inducer (Figure 1a, b). Robust induction of TotM was observed as early as 6 hours post α-actinin injection and was sustained above control levels for at least two days (Figure 1c). The ability of α-actinin to induce STAT-responsive gene induction was independently reproduced in three laboratories (C.R.S, M.D., L.T.), underscoring the robustness of the result (data not shown). Like actin itself, α-actinin is a component of the cytoskeleton in all higher eukaryotes where it crosslinks and stabilises actin filaments (Ribeiro et al., 2014). Given its close association with actin, α-actinin could therefore be present as a contaminant in
purified actin preparations, including the ones used in our studies. Consistent with that possibility, mass spectrometry analysis revealed that α-actinin is the major contaminant of purified actin preparations and accounts for approximately 0.4 % of total protein (Figure 1d). Western blot analysis revealed the presence of immunodetectable α-actinin in actin preparations, confirming the mass spectrometry results (Figure 1e). Notably, dose response curves showed that α-actinin was \( >100 \)-fold more potent than actin on a per molecule basis at eliciting the *Drosophila* STAT response (Figure 1f). Therefore, it is possible that contamination with α-actinin accounts for the activity of injected actin preparations in *Drosophila*.

To address this possibility, we tried to deplete α-actinin from actin preparations. However, we failed to satisfactorily separate actin and α-actinin using multiple approaches, including size exclusion chromatography, ionic strength chromatography or immunodepletion with 14 different antibodies (data not shown). We therefore pursued an alternative strategy of testing recombinant actin and α-actinin expressed in BL21 *E. coli*, an organism that does not have an actin-based cytoskeleton and in which, therefore, co-purification of α-actinin with actin is not possible. We first assessed the functional integrity of the recombinant proteins purified from bacteria. Using a pelleting assay (Ahrens et al., 2012), we confirmed that recombinant actin was able to form filaments when incubated in polymerisation-favouring conditions (Figure 2a) and that α-actinin was able to associate with those filaments (Figure 2b). Having ascertained that actin and α-actinin are functional and, therefore, correctly folded, we next tested whether they could elicit the expression of STAT-responsive genes in *Drosophila*. Strikingly, injection of bacterially-expressed actin did not elicit the expression of *TotM* or of another STAT target gene, *Diedel*, unlike actin purified from rabbit muscle (Figure 2c). In contrast, bacterially-expressed α-actinin, like α-
actinin purified from rabbit muscle, elicited the expression of various STAT-responsive genes, including TotM, TotA and Diedel, in a dose-dependent manner (Figure 2d and data not shown). Neither recombinant bacterially-expressed nor rabbit muscle derived α-actinin elicited the expression of Drs and Dpt, Toll and Imd target genes, respectively, 24h post injection (Figure 2 - figure supplement 1a), indicating that the proteins were devoid of microbial contaminants and confirming specificity for STAT targets. Lack of expression of Drs and Dpt by α-actinin was confirmed at multiple timepoints within a 48h period (Figure 2 - figure supplement 1b). In contrast, septic injury (Escherichia coli and Micrococcus luteus) induced both Drs and Dpt by 24h (Figure 2 - figure supplement 1c). Finally, RNAi-mediated knockdown of Nox, Src42A and Shark using three fat body-restricted drivers (c564, r4 and Lpp) abrogated the induction of STAT-responsive genes in response to injection of α-actinin (Figure 3a), whereas knockdown of Nox, Src42A and Shark using a haemocyte-restricted driver (HmlΔ) did not alter the response (Figure 3b). Therefore, injection of bacterially-expressed or rabbit muscle α-actinin into Drosophila melanogaster is sufficient to induce the same response that we previously described to be elicited by injection of actin preparations. Taken together, these data suggest that α-actinin is a major bioactive component in cytoskeletal protein preparations, including actin. We have previously reported that actin injection, mimicking extracellular cytoskeletal exposure, can trigger the expression of STAT target genes in Drosophila (Srinivasan et al., 2016). Actin is difficult to produce as a recombinant protein in bacteria and commercially-available actin (> 99% pure) is traditionally purified from rabbit muscle and human platelets. Here, we demonstrate that the activity of such actin preparations is likely attributable to trace amounts of co-purifying α-actinin. This conclusion is supported by several findings: 1) α-actinin engages the same fat body
Nox2/Src/Shark pathway previously described for injected actin; 2) contamination with α-actinin can account stoichiometrically for the activity of actin preparations, α-actinin being more than one-hundred fold more potent that actin; 3) recombinant α-actinin expressed in bacteria retains activity while activity is lost for bacterially-expressed actin. These new findings support our earlier conclusion that exposure of cytoskeletal components is an evolutionarily-conserved sign of cell damage but suggest that the nature of the cytoskeletal element acting as a DAMP can vary across species. Thus, while (F-) actin is sensed by at least one mammalian receptor, DNGR-1, in Drosophila melanogaster α-actinin appears to act as the dominant trigger for the STAT-dependent response we have studied. Consistent with the notion that α-actinin may be the common denominator of the response to extracellular proteins in Drosophila, preliminary mass spectrometry analysis suggests that trace amounts of α-actinin are also found in tubulin and myosin preparations (data not shown). The inability to deplete α-actinin using antibodies makes it difficult to address the issue of contamination, especially in the case of large multi-subunit proteins such as myosin for which recombinant expression in bacteria is not feasible. More work is needed to clarify whether any cytoskeletal proteins besides α-actinin act as inducers of STAT-responsive genes in Drosophila and to identify the putative receptor(s) responsible for initiating the response.
Materials and Methods

Fly maintenance and injections
Fly maintenance, breeding, transgene induction, injections, septic injury, RNA extraction, cDNA synthesis and quantitative real-time PCR were performed as previously described (Srinivasan et al., 2016).

The following stocks were used:

<table>
<thead>
<tr>
<th>Fly stock</th>
<th>Description</th>
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<tbody>
<tr>
<td>w^{1118}</td>
<td>Control strain</td>
</tr>
<tr>
<td>;UAS-Shark IR; (Shark Fr^{RNAi})</td>
<td>Interfering RNA for knockdown of shark. Kindly donated by Marc Freeman.</td>
</tr>
<tr>
<td>w^{c564-Gal4;Tub-Gal80{ts}}</td>
<td>Temperature-sensitive fat body-specific driver line.</td>
</tr>
<tr>
<td>;UAS-Src42A IR</td>
<td>VDRC ID: V100708</td>
</tr>
<tr>
<td>UAS-Nox IR</td>
<td>VDRC ID: 100753</td>
</tr>
<tr>
<td>;r4-Gal4/TM6C.Sb</td>
<td>Fat body-specific driver line</td>
</tr>
<tr>
<td>;Lpp-Gal4/TM6.Sb</td>
<td>Fat body-specific driver line</td>
</tr>
<tr>
<td>HmlΔ-Gal4/CyO</td>
<td>Haemocyte-specific driver</td>
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Purified cytoskeletal proteins
Purified cytoskeletal proteins were reconstituted and stored as advised by the supplier (Cytoskeleton Inc).

Bacterial expression of cytoskeletal proteins
Rosetta-2 BL21 E. coli bacteria were transformed with pET8c-His6-human Actinin-2 as described (Ribeiro et al., 2014). A starter culture was made by inoculating
Ampicillin-containing LB with a well-defined colony and incubating overnight at 37 °C. Large-scale expression was carried out using Overnight Express LB medium (Novagen) supplemented with 10 ml glycerol inoculated with 2 ml of the starter culture. Cell growth and protein expression was carried out at 30 °C for 16 hrs. Cells from these high-density shaking cultures (OD600 > 8 A.U.) were harvested by centrifugation for 15 min at 7500 x g. For purification of the His-tagged protein, the bacterial pellet was re-suspended in 50 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Triton X-100 and antiproteases (Roche). Cells were disrupted by sonication (5 x 30 seconds) and lysates were centrifuged at 18,000 x g for 30 min at 4 °C. Clarified lysates were incubated with 3 ml Ni-NTA agarose at 4 °C for 16 hrs on rotation. Beads were washed three times for 5 min at 4 °C with 25 ml wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, antiproteases, 5mM Imidazole). After the final wash, beads were incubated with 10 ml buffer A to elute His-tagged proteins (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, antiproteases, 2mM beta-mercaptoethanol, 250 - 500 mM Imidazole). Two elutions were collected and subsequently purified to homogeneity by anion exchange and size exclusion chromatography. To that end, α-actinin eluted from the Ni-NTA affinity resin was dialysed into buffer containing 50 mM Tris-HCl (pH 7.5), 2mM beta-mercaptoethanol and applied to a Mono Q 5/50 anion exchange column (GE Life Sciences) equilibrated with 50 mM Tris (pH 7.5) and 1 mM DTT. Bound α-actinin was eluted by application of a NaCl gradient from 0 to 1M over 30 column volumes. The peak containing α-actinin was determined by SDS-PAGE, the relevant fractions pooled and concentrated to 0.5 ml and applied to a S75 10/300 size exclusion column equilibrated with PBS (pH 7.4) and 1 mM DTT. Fractions containing α-actinin were
pooled and stored until required. Recombinant bacterially-expressed human smooth muscle actin was purchased from a commercial source (ab134555, Abcam).

**Mass spectrometry**

Protein preparations were subjected to SDS/PAGE and migrated approximately 2 cm into the gel. The gel lanes were excised and proteins were in-gel digested using trypsin. Tryptic peptides were analysed with an LTQ Orbitrap-Velos mass spectrometer coupled to an Ultimate3000 HPLC equipped with an EASY-Spray nanosource (Thermo Fisher Scientific). Raw data was processed using MaxQuant v1.3.05 with intensity based absolute quantification (iBAQ) selected as the quantification algorithm. The output table was imported into Perseus software v1.4.0.2, and percentages of contamination were calculated using the untransformed iBAQ values.

**Actin pelleting assay**

Performed as described by the supplier (Cytoskeleton). Briefly, actin was diluted in modified G-actin buffer (5 mM Tris HCl pH 8.0, 0.2 mM CaCl$_2$, 0.2 mM ATP, 0.5 mM DTT) and incubated on ice for 60 min for complete depolymerisation of actin oligomers. Subsequently, samples were supplemented with F-actin buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM MgCl$_2$ and 1 mM ATP) incubated for 60 min at RT. Samples were then centrifuged for 1 h at 100,000 x g, and protein levels in the supernatant and pellet were assessed by and coomassie staining. For α-actinin pelleting assays the same protocol was used, with the only modification that α-actinin was added to the polymerising actin filaments.
Western blotting

Protein samples were resuspended in 1X Laemmli SDS-PAGE buffer and boiled for 7 min at 95°C. Protein was resolved on Tris-Glycine 4-20% precast gels (Bio-Rad).

Actinin and actin was detected via immunoblotting with anti-actin (Clone C4, MAB1501, Millipore) and anti-actinin (3134S, Cell Signaling Technology) antibodies.

References


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Author Information The authors declare no competing financial interests.
Figure legends

Figure 1. α-actinin is the most potent inducer of STAT-dependent genes. (a) w^{1118} flies were injected with equimolar preparations of purified actin, α-actinin, myosin and tubulin (9.4 x 10^{17} molecules). Relative expression of TotM 24 h post injection is shown. Data are pooled from two independent experiments with 10 flies/sample with at least quadruplicate samples. (b) w^{1118} flies were injected with preparations containing the indicated number of molecules of purified myosin or purified α-actinin. Relative expression of TotM 24 h post injection is shown. Data are representative of two independent experiments with 10 flies/sample with duplicate samples. (c) w^{1118} flies were injected with buffer or 3.68 ng of purified α-actinin. Relative expression of TotM over a 48h period is shown. Data are representative of two independent experiments with 10 flies/sample with triplicate samples. (d) Purified actin was subjected to mass spectrometry analysis and contaminating proteins are expressed as % of protein preparation. (e) Indicated protein amounts (ng) of purified actin and α-actinin were analysed by western blot using an anti-α-actinin antibody. Data are representative of three independent experiments. (f) w^{1118} flies were injected with preparations containing the indicated number of molecules of rabbit muscle purified actin or purified α-actinin. Relative expression of TotM 24 h post injection is shown. Data are representative of three independent experiments with 10 flies/sample with duplicate samples.

TotM relative levels were calculated using the housekeeping gene Rp49 as a reference gene. Bars represent mean ± SD.
Figure 2. Bacterially-expressed recombinant α-actinin but not actin induces STAT-responsive genes. (a) Actin purified from rabbit muscle or recombinant made in bacteria was incubated in either G-actin or F-actin buffers before subjecting samples to ultracentrifugation to separate globular and filamentous actin. Supernatant (S) and pelleted (P) fractions were analysed by Tris-Glycine gels and visualized by coomassie staining. Data are representative of two independent experiments. (b) α-actinin from rabbit muscle or recombinant made in bacteria was added or not to polymerised F-actin. Then, samples were subjected to ultracentrifugation, and proteins in the supernatant (S) or pellet (P) were analysed by Tris-Glycine gels and visualized by coomassie staining. (c) w1118 flies were injected with PBS buffer or equal amounts of bacterially-expressed recombinant or rabbit muscle purified actin (11.04ng per fly). Relative expression of TotM and Diedel 24 h post injection is shown. Data are representative of two independent experiments with 10 flies/sample with triplicate samples. (d) w1118 flies were injected with PBS buffer, or equal amounts of bacterially-expressed recombinant or rabbit muscle purified α-actinin (3680pg or 368pg per fly). Relative expression of TotM and TotA 24 h post injection is shown. Data are representative of two independent experiments with 10 flies/sample with triplicate samples.

TotM relative levels were calculated using the housekeeping gene Rp49 as a reference gene. Bars represent mean ± SD. Statistical analysis was performed using one-way ANOVA with Sidak’s multiple comparison test as post-test for pairwise comparisons. Significant differences with Sidak’s multiple comparison test are shown (ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).
Figure 3. α-actinin induced STAT-dependent genes require Nox, Shark and Src42A expression in the fat body. (a) Flies in which Shark, Nox, or Src42A were knocked down in the fat body using three different driver lines (c564-Gal80b, r4-Gal4 or Lpp-Gal4), or control flies lacking UAS-target sequences, were injected with either PBS buffer or rabbit muscle purified α-actinin (13.8pg per fly). Relative expression of TotM 24 h post injection is shown. Data are representative of two independent experiments with 5-10 flies/sample with at least triplicate samples. (b) Flies in which Shark, Nox, or Src42A were knocked down in haemocytes (HmlΔ-Gal4), or control flies lacking UAS-target sequences, were injected with either PBS buffer or rabbit muscle purified α-actinin (13.8pg per fly). Relative expression of TotM 24 h post injection is shown. Data are representative of two independent experiments with 5-10 flies/sample with triplicate samples.

TotM relative levels were calculated using the housekeeping gene Rp49 as a reference gene. Bars represent mean ± SD. Statistical analysis was performed using one-way ANOVA with Sidak’s multiple comparison test as post-test for pairwise comparisons. Significant differences with Sidak’s multiple comparison test are shown (ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).
Figure 2 – Figure Supplement 1. Bacterially-expressed recombinant or rabbit muscle purified α-actinin do not induce the expression of anti-microbial genes. (a) w^{1118} flies were injected with PBS buffer, or equal amounts of bacterially-expressed recombinant or rabbit muscle purified α-actinin (3.68ng or 368pg per fly). Relative expression of Drosomycin (Drs) and Dipterisin (Dpt) 24h post injection is shown. Data are representative of two independent experiments with 10 flies/sample with triplicate samples. (b) w^{1118} flies were injected with PBS buffer or 3.68ng rabbit muscle purified α-actinin and relative expression of Drs and Dpt monitored over a 48h timeframe. (c) w^{1118} flies were subjected to injury with a fine needle that was either previously dipped in PBS (buffer) or a concentrated mixture of M. luteus and E. coli (septic injury). Relative expression of Drs and Dpt 24h after injury is shown. Data are representative of two independent experiments with 10 flies/sample with quadruplicate samples. Drs and Dpt relative levels were calculated using the housekeeping gene Rp49 as a reference gene. Bars represent mean ± SD.
**Figure 1**

(a) Bar charts showing the relative mRNA/Rp49 for Actin, Myosin, Actinin, and Tubulin.

(b) Graphs depicting the relative mRNA/Rp49 for Actin and Myosin against the number of molecules (x10^10).

(c) Graph showing the relative mRNA/Rp49 for TotM against time (h).

(d) Bar chart illustrating the protein content in Actin preparations.

(e) Western blot analysis for Actin and Actinin with different concentrations of Buffer and MW (kDa).

(f) Graph comparing the relative mRNA/Rp49 for TotM against the number of molecules (x10^10).
Figure 3

**a**

- **TotM**
  - c564-Gal80
  - w^{1118}
  - UAS-Shark IR
  - UAS-Nox IR
  - UAS-Src42A IR

- **TotM**
  - Lpp-Gal4
  - w^{1118}
  - UAS-Shark IR
  - UAS-Nox IR
  - UAS-Src42A IR

**b**

- **TotM**
  - Hmlα-Gal4
  - w^{1118}
  - UAS-Shark IR
  - UAS-Nox IR
  - UAS-Src42A IR

Legend:
- ****: p < 0.0001
- ***: p < 0.001
- ****: p < 0.01
- *: p < 0.05
- ns: not significant
Figure 2 - figure supplement 1

(a) Drs and Dpt relative mRNA/Rp49 levels in different buffers and purified Actinin.

(b) Drs and Dpt relative mRNA/Rp49 levels over time (hrs) with different buffers and Actinin.

(c) Drs and Dpt relative mRNA/Rp49 levels in Buffer and Septic Injury conditions.