The c-src Homologue *Src64B* Is Sufficient to Activate the *Drosophila* Cellular Immune Response

Michael J. Williams

Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, UK

Key Words

Innate immunity • Tyrosine kinase • Haemocyte • Src

Abstract

The *Drosophila* larval cellular immune response involves cells (haemocytes) that can be recruited from a haematopoietic organ known as the lymph gland, as well as a sessile population of cells found just underneath the larval cuticle arranged in a segmental pattern. Overexpression of the *Drosophila* c-src homologue *Src64B* disrupts the sessile-haemocyte banding pattern. Though *Src64B* overexpression induced the formation of specialized haemocytes known as lamellocytes, its hyperactivation had no effect on circulating plasmatocyte concentration. Also, there is evidence of non-autonomous regulation as *Src64B* activity was observed in non-overexpressing plasmatocytes. Finally, *Src64B* overexpression induced F-actin formation and Jun kinase activation in plasmatocytes.

Copyright © 2009 S. Karger AG, Basel

Introduction

The *Drosophila melanogaster* larval cellular immune response involves circulating immunosurveillance cells known as haemocytes. In *Drosophila* larvae, haemocytes are located in three main compartments: in circulation, in a haematopoietic organ known as the lymph gland, and as a sessile-haemocyte population found just underneath the

larval cuticle [1–4]. Based on morphology, three basic types of haemocytes can be identified: plasmatocytes, lamellocytes and crystal cells. In a healthy larva, the most abundant circulating haemocytes are the plasmatocytes, small cells involved in phagocytosis and able to produce antimicrobial peptides. The largest and normally least abundant haemocytes are the lamellocytes, involved in the encapsulation of invading pathogens [5–7]. Crystal cells rupture upon activation to release components of the phenol oxidase cascade, which is involved in melanisation of invading organisms, wound repair and coagulation [2, 8, 9].

Src family tyrosine kinases are composed of a tyrosine kinase domain, an SH2 domain, an SH3 domain, and a regulatory C-terminal region. They are known to be activated by receptor tyrosine kinases, G-protein-coupled receptors, and integrins, and are involved in regulating cell cycle, cytoskeletal rearrangements, and cell adhesion [10, 11]. The *Drosophila* genome contains two Src family tyrosine kinases, *Src42A* and *Src64B* that are functionally similar to their mammalian counterparts fyn-related kinase (FRK) and c-src, respectively. *Src42A* and *Src64B* loss-of-function mutations disrupt cytoskeletal regulation within developing oocytes and embryos [12–14]. Yet the full repertoire of src family tyrosine kinase function remains to be elucidated.

The current study shows that *Src64B* is able to activate the *Drosophila* cellular immune response. Overexpression of *Src64B* specifically in haemocytes disrupts the larval haemocyte segmental-banding pattern and induces lamellocyte formation.

Fig. 1. Src64B overexpression disrupts the sessile-haemocyte banding pattern. **a** GFP expression in sessile-haemocytes of control larvae (*He-Gal4*) and larvae expressing wild-type Src64B (*UAS-Src64B/He-Gal4, UAS-GFPnls*). **b** Circulating haemocyte counts after overexpression of Src64B. *He-Gal4* was crossed with *UAS-Src64B*. Haemocytes were counted from at least 15 individual larvae. * Indicates significant difference (Student's t test, $p < 0.01$) compared with the parental *UAS* and *He-Gal4* strains.

Methods

Insects

Drosophila strain *UAS-Src64B* was obtained from the Bloomington Stock Center. The *Hemese-GAL4, UAS-GFPnls* driver line has been described previously [15]. Flies were kept on a standard cornmeal diet (60% w/v yellow cornmeal, 12% w/v inactive dry yeast, 7.5% v/v golden syrup, 1.4% w/v methyl paraben (Tegosept), and 6% w/v agar) at between 21 and 25°C. Stocks crossed to *GAL4* driver flies and the uncrossed control flies were raised at 29°C.

Immunofluorescence

For all antibody staining, haemocytes were bled from larvae into 20 µl of phosphate-buffered saline (PBS) and allowed to attach to a glass slide (SM-011, Hendley-Essex, Essex, UK) for 1 h. Staining and analysis were done according to Williams et al. [16]. The polyclonal anti-phospho-JNK (Promega) was diluted 1:500 in 3% BSA/PBS. The polyclonal anti-phospho-Src64B [17] was diluted 1:500 in 3% BSA/PBS. Alexa Fluor 488-phalloidin (Molecular Probes) was diluted to a final concentration of 0.20 µg in 1% BSA/PBS. Nuclei were stained by incubating the cells with DAPI diluted 1:5,000 in 1× PBS. Cells were visualized using a Zeiss Axiovert 200M epifluorescent microscope and digital pictures were taken with a Hamamatsu C4742-80-12AG video unit, controlled by the Simple PCI 6.1 program (Hamamatsu). ImageJ (NIH) was used for digital editing. ImageJ was used to measure fluorescence intensity.

To visualize haemocyte patterns within larvae, wandering third-instar larvae were washed in PBS and then killed by freezing at -80°C for 1.5 min. The larvae were then transferred to a glass slide and covered in 50% glycerol and visualized as described previously [4, 16].

Haemocyte Counting and Statistics

Haemocyte counting and statistics were done as described previously [4]. Briefly, *UAS* transgenic lines were crossed to *Hemese-Gal4, UAS-GFPnls*. The females were allowed to lay eggs at 21–25°C for 2 days before the vials were moved to 29°C. Larvae were staged according to procedures described by Andres and Thummel [18]. Staged larvae were washed in PBS before being

bled into 20 µl of PBS, by using a pair of fine forceps and a 27-gauge needle. The PBS containing the haemocytes was then loaded onto a Neubauer improved haemocytometer for counting. Haemocytes from at least 15 larvae from each strain were counted, and statistical analysis was done according to the procedures outlined previously [4].

Measurement of Cell Size

Haemocytes were bled from larvae into 20 µl of PBS and allowed to attach to a glass slide (SM-011, Hendley-Essex, Essex, UK) for 1 h. The cells were then stained with Cy2-phalloidin as stated previously. F-actin was visualized using epifluorescence and digital pictures were taken with a Hamamatsu C4742-80-12AG video unit, controlled by the Simple PCI 6.1 program (Hamamatsu). Cell area measurements were made by measuring the cells on the x- and y-axes using the ImageJ program and taking the average of these two measurements in micrometres. For statistics, an initial ANOVA (<http://www.physics.csbsju.edu/stats/anova.html>) indicated that the overexpression of the *UAS* constructs significantly affect haemocyte cell size. Multiple Student's t tests (Microsoft Excel and <http://www.graphpad.com/quickcalcs/ttest1.cfm>) were performed to study specific interactions between certain genotypes and their corresponding crosses.

Results and Discussion

To begin to study the influence of Src tyrosine kinase signalling on *Drosophila* haemocytes, *UAS-Src64B* transgenic flies were crossed to the haemocyte-specific driver line *Hemese-GAL4, UAS-GFPnls* (hereafter called *He-Gal4*). In third-instar control larvae, segmentally arranged haemocytes were observed just underneath the cuticle (fig. 1a, arrows). Overexpression of wild-type Src64B disrupted the haemocyte segmental-banding pattern (fig. 1a, arrows).

Fig. 2. Src64B overexpression is sufficient to activate JNK in plasmatocytes. **a** Haemocytes were recovered from early third-instar larvae and stained for Bsk activation using anti-phosphorylated-JNK antibody (red). Cells were counter-stained with Cy2-phalloidin to visualize their actin cytoskeleton (green). In the merged pictures, overlap of the two stainings is yellow. **b** JNK activity. Quantification of Bsk activity. ImageJ was used to measure fluorescence intensity of at least 100 haemocytes from 3 different larvae. * Significant difference (Student's t test, $p < 0.01$) compared with the parental *UAS* and *He-Gal4* strains. **c** Quantification of F-actin expression levels. Haemocytes were bled from wandering

third-instar larvae and stained with Cy2-phalloidin. ImageJ was used to measure fluorescence intensity of at least 100 haemocytes from 3 different larvae. * Significant difference (Student's t test, $p < 0.01$) compared with the parental *UAS* and *He-Gal4* strains. **d** Determination of plasmatocyte size. The cell diameter of plasmatocytes from at least 100 haemocytes from at least 3 different larvae per genotype was measured, as described in Materials and Methods, and the diameter (micrometres) was plotted. * Significant difference (Student's t test, $p < 0.01$) compared with the parental *UAS* and *He-Gal4* strains.

In most instances, disruption of the sessile banding pattern leads to an increase in the number of circulating haemocytes [4]. While overexpression of Src64B had no effect on the number of circulating plasmatocytes, there was a significant increase in the number of circulating lamellocytes (fig. 1b). This increase in the number of circulating lamellocytes was not at the expense of the plasmatocyte population, meaning an overall 2-fold increase in the total number of circulating haemocytes was observed compared to control larvae. Previously, in a di-

rected screen to discover genes involved in regulating the *Drosophila* cellular immune response, only overexpression of *hop^{tum1}*, a constitutive active version of the *Drosophila* JAK kinase *hopscotch*, was able to induce lamellocyte formation without causing a change in the number of circulating plasmatocytes [4]. In mammalian tissue culture models, it has been shown that src tyrosine kinase can directly phosphorylate and activate the transcription factor STAT3 in vitro, and STAT3 function and activation are required for src transforming activity in multiple

Fig. 3. Src64B activity may be cell non-autonomous. Haemocytes were bled from early third-instar *UAS-Src64B;He-Gal4, UAS-GFPnls* larvae and stained with anti-phospho-Src64B (red). GFP expression (green). Arrows show cell non-autonomous activation of Src64B in plasmatocytes. Open arrows show low level of Src64B in a lamellocyte. Arrowheads high level of Src64B activity in a GFP-minus plasmatocyte.

tissue culture cell lines [19–21]. STAT activity is also known to be regulated by JAK kinases in both mammals and *Drosophila* [22–25]. Furthermore, in the *Drosophila* eye, Src-regulated signalling pathways were shown to require STAT activity [26]. From these results I conclude that *Src64B* overexpression is sufficient to activate *Drosophila* circulating haemocytes. Since overexpression of *Src64B* in haemocytes has a similar phenotype to *hop^{tum1}*, it is possible that *Src64B* and the JAK/STAT pathway interact in haemocytes.

In *Drosophila*, *Src64B* has been shown to activate JNK signalling and regulate cytoskeletal dynamics [12, 27]. To see if the same could be true for the cellular immune response, haemocytes were bled from third-instar control and *UAS-Src64B;He-Gal4* larvae and stained with an anti-phospho-JNK antibody to visualize active JNK, as well as Cy3-phalloidin to observe the actin cytoskeleton. Haemocytes from control larvae were round in appearance with little F-actin at the plasma membrane (fig. 2a). When haemocytes were bled from control larvae (*He-Gal4* or *UAS-Src64B*) and stained with anti-phospho-JNK, staining was observed at the periphery of the cell, in the nucleus, and as punctate points of activity between the nucleus and cell periphery (fig. 2a, and data not shown). Overexpression of wild-type *Src64B* induced a significant increase, 2.5-fold, in the amount of active JNK in plasmatocytes (fig. 2b). Though there were many lamellocytes present in the haemocyte bleeds from *UAS-Src64B;He-Gal4* larvae, very little active JNK was observed in lamellocytes. In a few lamellocytes JNK activity co-localized with DAPI staining in the nucleus (fig. 2a, arrows, DAPI staining not shown).

When compared to haemocytes from control larvae, overexpression of wild-type *Src64B* induced a significant increase, 4.4-fold, in F-actin (fig. 2c). Also similar to what is observed in plasmatocytes from parasitized larvae [28], cell diameter seemed to correlate with F-actin formation. Control haemocytes had an average diameter of 22 µm, while haemocytes that were overexpressing *Src64B* had an average diameter of 58 µm. Furthermore, many *Src64B* overexpressing plasmatocytes had extremely ruffled membranes (fig. 2a, Detail). All of these phenotypes are similar to haemocytes recovered from parasitized larvae [28].

To examine in which cell subtypes *Src64B* may be active, haemocytes were bled from third-instar *He-Gal4* and *UAS-Src64B* controls or *UAS-Src64B;He-Gal4* larvae and stained with an antibody that recognizes active *Src64B* (anti-phospho-*Src64B*) [17]. The *He-Gal4* construct is only active in about 80% of circulating haemocytes [4]. When *Src64B* was overexpressed in haemocytes, *Src* activity was observed in both GFP-positive and -negative plasmatocytes (fig. 3, arrows). Also, most lamellocytes had no *Src* activity; if activity was observed (fig. 3, open arrows), it was at much lower levels than in plasmatocytes (fig. 3, arrowheads). No *Src64B* activity was observed in haemocytes bled from third-instar control larvae (data not shown). The presence of active *Src64B* in GFP-negative plasmatocytes could mean that *Src* overexpression in some plasmatocytes is able to somehow signal to non-overexpressing cells leading to the activation of endogenous *Src64B* in a cell non-autonomous manner. Even though *He-Gal4*, and thus *UAS-Src64B*, is expressed in all haemocyte subtypes, activated *Src64B* was rarely

observed in lamellocytes. This is evidence that overexpression of Src64B is not sufficient for its activation in some cell types.

This study shows that similar to the *Drosophila* JAK kinase *hopscotch*, the c-src homologue Src64B induces lamellocyte differentiation without increasing circulating plasmatocyte concentration. Also, Src64B overexpression was able to induce JNK activation in haemocytes. Similar to what was reported by Read et al. [26] in imaginal discs, this may be evidence of Src64B interacting with the JAK/STAT and JNK pathways in haemocytes. Src64B, which disrupts the sessile haemocyte banding pattern when overexpressed specifically in haemocytes, induces both actin formation and JNK activation. Previously, we reported that the small GTPase Rac1 requires both actin formation as well as JNK activation to

disrupt the sessile haemocyte banding pattern. The same may be true for Src64B activity in haemocytes. Finally, this study may show cell-cell signalling occurs in haemocytes during cellular activation as activated Src64B was observed in many plasmatocytes not overexpressing Src64B.

Acknowledgements

I would like to thank Dr. Michael Simon for his kind gift of anti-phospho-Src64B antibody. This research was supported in part by Dan Hultmark's grants from the Swedish Research Council and the Swedish Cancer Society. It was also supported by funds provided by the Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, UK.

References

- Crozatier M, Meister M: *Drosophila* haematopoiesis. Cell Microbiol 2007;9:1117–1126.
- Williams MJ: *Drosophila* hemopoiesis and cellular immunity. J Immunol 2007;178:4711–4716.
- Goto A, Kadowaki T, Kitagawa Y: *Drosophila* hemolymph gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. Dev Biol 2003;264:582–591.
- Zettervall C, et al: A directed screen for genes involved in *Drosophila* blood cell activation. Proc Natl Acad Sci USA 2004;101:14192–14197.
- Carton Y, Nappi AJ: *Drosophila* cellular immunity against parasitoids. Parasitol Today 1997;13:218–227.
- Lanot R, Zachary D, Holder F, Meister M: Postembryonic hematopoiesis in *Drosophila*. Dev Biol 2001;230:243–257.
- Sorrentino RP, Carton Y, Govind S: Cellular immune response to parasite infection in the *Drosophila* lymph gland is developmentally regulated. Dev Biol 2002;243:65–80.
- Lemaitre B, Hoffmann J: The host defense of *Drosophila melanogaster*. Annu Rev Immunol 2007;25:697–743.
- Bidla G, Dushay MS, Theopold U: Crystal cell rupture after injury in *Drosophila* requires the JNK pathway, small GTPases and the TNF homolog *eiger*. J Cell Sci 2007;120:1209–1215.
- Ingle E: Src family kinases: regulation of their activities, levels and identification of new pathways. Biochim Biophys Acta 2008;1784:56–65.
- Li S, Shang Y: Regulation of Src family co-activators by post-translational modifications. Cell Signal 2007;19:1101–1112.
- Djagaeva I, Doronkin S, Beckendorf SK: Src64 is involved in fusome development and karyosome formation during *Drosophila* oogenesis. Dev Biol 2005;284:143–156.
- Shindo M, et al: Dual function of Src in the maintenance of adherens junctions during tracheal epithelial morphogenesis. Development 2008;135:1355–1364.
- Murray MJ, Davidson CM, Hayward NM, Brand AH: The Fes/Fer non-receptor tyrosine kinase cooperates with Src42A to regulate dorsal closure in *Drosophila*. Development 2006;133:3063–3073.
- Keely PJ, Westwick JK, Whitehead IP, Der CJ, Parise LV: Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. Nature 1997;390:632–636.
- Williams M, Wiklund M, Wikman S, Hultmark D: Rac1 signalling in the *Drosophila* larval cellular immune response. J Cell Sci 2006;119:2015–2024.
- O'Reilly AM, et al: Csk differentially regulates Src64 during distinct morphological events in *Drosophila* germ cells. Development 2006;133:2627–2638.
- Andres A, Thummel C: Methods for quantitative analysis of transcription in larvae and prepupae. Methods Cell Biol 1994;44:565–573.
- Han C, Bowen WC, Michalopoulos GK, Wu T: α -adrenergic receptor transactivates signal transducer and activator of transcription-3 (Stat3) through activation of src and epidermal growth factor receptor (EGFR) in hepatocytes. J Cell Physiol 2008;DOI 10.1002/jcp.21420.
- Haura EB: SRC and STAT pathways. J Thorac Oncol 2006;1:403–405.
- Bromberg JF, Horvath CM, Besser D, Lathem WW, Darnell JE, Jr: Stat3 activation is required for cellular transformation by v-src. Mol Cell Biol 1998;18:2553–2558.
- Luo H, Hanratty WP, Dearolf CR: An amino acid substitution in the *Drosophila* hopTum-1 Jak kinase causes leukemia-like hematopoietic defects. EMBO J 1995;14:1412–1420.
- Harrison DA, Binari R, Nahreini TS, Gilman M, Perrimon N: Activation of a *Drosophila* Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. EMBO J 1995;14:2857–2865.
- Cao X, Tay A, Guy GR, Tan YH: Activation and association of Stat3 with src in v-src-transformed cell lines. Mol Cell Biol 1996;16:1595–1603.
- Sorrentino RP, Melk JP, Govind S: Genetic analysis of contributions of dorsal group and JAK-Stat92E pathway genes to larval hemocyte concentration and the egg encapsulation response in *Drosophila*. Genetics 2004;166:1343–1356.
- Read RD, Bach EA, Cagan RL: *Drosophila* C-terminal Src kinase negatively regulates organ growth and cell proliferation through inhibition of the Src, Jun N-terminal kinase, and STAT pathways. Mol Cell Biol 2004;24:6676–6689.
- Tateno M, Nishida Y, Adachi-Yamada T: Regulation of JNK by Src during *Drosophila* development. Science 2000;287:324–327.
- Williams M, Ando I, Hultmark D: *Drosophila melanogaster* Rac2 is necessary for a proper cellular immune response. Genes Cells 2005;10:813–823.