Developmental and Comparative Immunology 38 (2012) 160–168

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

The RhoGEF Zizimin-related acts in the *Drosophila* cellular immune response via the Rho GTPases *Rac2* and *Cdc42*

Christopher J. Sampson [a,1], Susanna Valanne [b], Marie-Odile Fauvarque [c,d,e], Dan Hultmark [b,f], Mika Rämet [b,g], Michael J. Williams [a,*]

[a] *Institute of Biological and Environmental Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen, UK*
[b] *Institute of Biomedical Technology, and BioMediTech, University of Tampere, Finland*
[c] *CEA, iRTSV, LBGE, Institut de Recherches en Technologies et Sciences pour le Vivant, F-38054 Grenoble, France*
[d] *INSERM, U1038, F-38054 Grenoble, France*
[e] *UJF-Grenoble 1, F-38041 Grenoble, France*
[f] *Department of Molecular Biology, Umeå University, Umeå, Sweden*
[g] *Department of Pediatrics, Tampere University Hospital, Finland*

ARTICLE INFO

Article history:
Received 14 March 2012
Revised 10 May 2012
Accepted 15 May 2012
Available online 23 May 2012

Keywords:
Haemocyte
Small GTPase
Innate immunity
Cellular immunity
Rho GTPase
Parasitization
Phagocytosis

ABSTRACT

Zizimin-related (*Zir*), a Rho guanine nucleotide exchange factor (RhoGEF) homologous to the mammalian Dock-C/Zizimin-related family, was identified in a screen to find new genes involved in the *Drosophila melanogaster* cellular immune response against eggs from the parasitoid wasp *Leptopilina boulardi*. RhoGEFs activate Rho-family GTPases, which are known to be central regulators of cell migration, spreading and polarity. When a parasitoid wasp is recognized as foreign, multiple layers of circulating immunosurveillance cells (haemocytes) should attach to the egg. In *Zir* mutants this process is disrupted and lamellocytes, a haemocyte subtype, fail to properly encapsulate the wasp egg. Furthermore, macrophage-like plasmatocytes exhibit a strong reduction in their ability to phagocytise *Escherichia coli* and *Staphylococcus aureus* bacteria. During encapsulation and phagocytosis *Zir* genetically interacts with two Rho-family GTPases, *Rac2* and *Cdc42*. Finally, *Zir* is dispensable for the humoral immune response against bacteria. We propose that *Zir* is necessary to activate the Rho-family GTPases *Rac2* and *Cdc42* during the *Drosophila* cellular immune response.

1. Introduction

When the morphology of *Drosophila* circulating immune surveillance cells (haemocytes) is compared, three cell types can be identified: plasmatocytes, crystal cells and lamellocytes (Fauvarque and Williams, 2011; Lemaitre and Hoffmann, 2007; Rizki and Rizki, 1984, 1992). Upon pathogenic invasion, haemocytes are recruited from a haematopoietic organ known as the lymph gland, as well as from a sessile-haemocyte population found in each segment of the larvae (Irving et al., 2005; Markus et al., 2009; Zettervall et al., 2004). Plasmatocytes, which are centrally involved in phagocytosis, encapsulation and the production of antimicrobial peptides, resemble the mammalian monocyte/macrophage lineage. In healthy larvae, plasmatocytes make up about ninety-five percent of all circulating haemocytes. The other approximately five percent of circulating haemocytes in healthy larvae consist of crystal cells, which rupture to secrete components of the prophenoloxidase cascade. Crystal cells are involved in melanization of invading organisms, wound repair and coagulation (Bidla et al., 2007; Lemaitre and Hoffmann, 2007; Williams, 2007). The third cell type, known as lamellocytes, are rarely seen in healthy larvae and seem to be specialized for the encapsulation of invading pathogens (Lanot et al., 2001; Rizki and Rizki, 1992; Sorrentino et al., 2004). Recent evidence reveals that a subpopulation of lamellocytes derives directly from circulating plasmatocytes (Honti et al., 2010).

Endoparasitic wasps from the Hymenoptera order parasitize *Drosophila* by laying an egg within the larval open circulatory system, in a cavity known as the hemocoel. Once a wasp egg is recognized as foreign, haemocytes adhere to the invader. After spreading around the wasp egg, plasmatocytes form cellular junctions between the cells, effectively separating the egg from the hemocoel (Fauvarque and Williams, 2011; Russo et al., 1996; Williams, 2009; Williams et al., 2005). Next, lamellocytes recognize the plasmatocytes attached to the egg. The final phase of encapsulation includes melanization of the capsule due to crystal cell rupture (Lemaitre and Hoffmann, 2007; Williams, 2007). From these events it is obvious that adhesion and cell shape change are essential

* Corresponding author. Current address: Functional Pharmacology, Department of Neuroscience, Uppsala University, Uppsala, Sweden. Tel.: +46 18 471 4159; fax: +46 18 46 511 540.
E-mail address: michael.williams@neuro.uu.se (M.J. Williams).
[1] Present address: School of Life Sciences, JMS Building, University of Sussex, Falmer, Brighton, United Kingdom.

0145-305X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2012.05.004

components of the *Drosophila* cellular immune response against parasitoid wasp eggs. One family of proteins central to these processes is the Rho-family GTPases (Fauvarque and Williams, 2011). Previously, we have reported that the Rho-family members *Rac1* and *Rac2* are essential for the *Drosophila* cellular immune response to react properly against eggs from *Leptopilina boulardi* (Avet-Rochex et al., 2007; Williams et al., 2005, 2006).

Rho-family GTPases are small GTP-binding proteins centrally involved in cytoskeletal rearrangement, cell adhesion and polarity (Bokoch, 2005; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004; Ridley, 2001). Upon signal transduction, small GTPases are activated when guanine nucleotide exchange factors (GEFs) promote exchange of GDP for GTP. RhoGEFs fall into either the Dbl-homology or Dock subfamilies. Dbl-homology family GEFs share a common motif responsible for catalyzing the exchange of nucleotides on Rho GTPases, known as a Dbl-homology (DH) domain. Dock-family RhoGEFs were initially categorized as atypical GEFs, as they contained no obvious catalytic domain for nucleotide exchange. Dock-family GEFs contain two conserved domains, deemed Dock homology region-1 (DHR-1) and Dock homology region-2 (DHR-2). The DHR-2 domain can bind and induce nucleotide exchange on the Rho-family member *Rac1* (Brugnera et al., 2002; Cote and Vuori, 2002, 2006), the DHR-1 region was shown to bind PtdIns(3,4,5)P(3) and is also involved in *Rac1* activation (Cote et al., 2005). The human genome has 11 Dock-type genes that breakdown into A, B, C and D subfamilies. Most members of the Dock-A and -B subfamilies, including *Drosophila myoblast city*, are specific *Rac* GTPase activators, while Dock-D/Zizimin subfamily members Dock9 and -11 have specificity for *Cdc42* (Kwofie and Skowronski, 2007). The Dock-C/Zizimin-related subfamily consists of Dock6, -7, and -8. Using the yeast two-hybrid system Ruusala and Aspenstrom (2004) reported that Dock8 interacts with both *Rac1* and *Cdc42*, though it is not known whether it can activate both GTPases. Dock6 was identified as the first Dock-type GEF having the ability to activate both *Rac1* and *Cdc42* (Miyamoto et al., 2007). On the other hand, Dock7 can interact with *Rac1* and *Rac3*, but does not interact with *Cdc42* (Watabe-Uchida et al., 2006).

We identified the *Drosophila* gene *CG11376*, a Dock-C/Zizimin-related homologue, as being necessary for both the proper encapsulation of eggs from the parasitoid wasp *L. boulardi* and for bacterial phagocytosis. Furthermore, similar to other Dock-C members, the *Drosophila* homologue seems to genetically interact with at least two Rho-family GTPases.

2. Materials and methods

2.1. Insects

Drosophila strain $CG11376^{BG00267}$ was obtained from the Bloomington Stock Center. *UAS-*$CG11376^{IR}$ strain number 40673 was obtained from the Vienna *Drosophila* RNAi Center (VDRC). *Hemese-GAL4* was described previously (Zettervall et al., 2004). Flies were kept on a standard cornmeal diet (60% w/v yellow cornmeal, 12% w/v inactive dry yeast, 7.5% w/v inverted 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. The G486 strain of *L. boulardi* was bred on a w^{1118} stock of *Drosophila melanogaster* at room temperature using standard medium. Adult wasps were maintained at room temperature in vials containing grape juice agar.

2.2. Wasp egg encapsulation assay

Encapsulation assays were done according to Sorrentino et al. (2002). Briefly, 2 days before parasitization the appropriate fly strains were crossed and kept at 21–25 °C. Four or five females of *L. boulardi* G486 were allowed to infest at room temperature for 2 h, after which the *Drosophila* larvae were left at room temperature for 38–40 h. After this time the larvae were collected, washed in PBS and analysed under a stereomicroscope for the presence of a dark capsule. Larvae not obviously carrying darkened capsules were dissected in 20 μl of phosphate buffered saline (PBS) to determine whether they had been parasitized. Larvae containing eggs of the parasitoid that had not darkened due to melanization by this time were scored as non-encapsulated. Non-parasitized larvae were excluded from the count.

2.3. Western analysis

Wandering third instar larvae were homogenized in cell lysis buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.3 M NaCl, 2% IGEPAL). Lysates were centrifuged at 8000×*g* for 5 min at 4 °C, supernatants were recovered to a new tube. Concentration was quantified using BioRad DC Protein Assay Kit (BioRad). Protein fractions (10 μg) were diluted with Laemmli buffer, heated at 65 °C for 10 min, and SDS–PAGE was performed using 4% polyacrylamide stacking/10% resolving gels. Rabbit anti-Dock7 (Watabe-Uchida et al., 2006) was diluted 1:1000, HRP-conjugated anti-rabbit secondary (Amersham) was diluted 1:10,000.

2.4. Immunofluorescence

2.4.1. Haemocytes on wasp eggs

Eggs 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 5 min at room temperature. Staining/analysis was done according to Williams et al. (2005). Lamellocyte monoclonal antibody (anti-L1a) and plasmatocyte monoclonal antibody (anti-Nimrod) were used undiluted (Kurucz et al., 2003, 2007).

2.5. Ex vivo phagocytosis tests on isolated third instar larvae plasmatocytes

Ex vivo phagocytosis tests were performed essentially as described in (Avet-Rochex et al., 2007; Pearson et al., 2003). The average phagocytic index per strain was a product of 100 individual phagocytic indices taken from 100 larvae. These larvae were seperated into groups of 5 larvae over 20 replicates in a 96-well plate. After the phagocytosis index assay was conducted, 5 separate images were captured at 20× magnification using a Zeiss N-achroplan objective at 5 seperate foci within a single well. This meant that a single image was a snap shot of phagocytosis per larva (a single well held 5 larvae therefore 5 images were taken creating a single image per larva). To create the phagocytic index (PI) the images captured were analysed using freeware ImageJ v1.41. Firstly the image, in RGB format, was converted into an RGB composite image where the green channel only was selected. This channel visualised the FITC-tagged Gram− or Gram+ bacteria. This was then converted into an 8-bit greyscale image, which underwent a tresholding to create a stark black over white contrast image (black dots (FITC-bacterial particles) over a white background). Finally, ImageJ particle analysis tool, with set parameters for circularity (0.3–1) and diameter in pixels (0.02–3), was used to count the number of bioparticles (black dots). This number now formed the total number of bacteria in that image. Finally, the number of cells containing bacteria, per image, was physically counted and this constituted the total number of cells. This was done on the original RGB image. PI was then calculated as the number of bacteria/number of cells. This was performed for every single image, yielding an *N* of 100 individual PI's, which was then pooled together to create the average PI.