Rac1 signalling in the *Drosophila* larval cellular immune response

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Summary

The *Drosophila* larval cellular immune response involves cells (hemocytes) that can be recruited from a hematopoietic organ located behind the brain, as well as a sessile population of cells found just underneath the larval cuticle arranged in a segmental pattern. By using two Rac1 GTPase effector-loop mutants together with epistasis studies, we show that Rac1 requires the *Drosophila melanogaster* Jun N-terminal kinase Basket (Bsk), as well as stable actin formation to recruit the sessile hemocyte population. We show that actin stabilization is necessary for Rac1-induced hemocyte activation by lowering cofilin (encoded by the twinstar gene *tsr*) expression in blood cells. Removing Bsk by RNAi suppressed Rac1-induced release of sessile hemocytes. RNAi against Bsk also suppressed Rac1 induction of lamellocytes, a specialized population of hemocytes necessary for the encapsulation of invading pathogens. Furthermore, Rac1 and Bsk are involved in regulating the formation of actin- and focal adhesion kinase (FAK)-rich placodes in hemocytes. Lastly, Rac1 and Bsk are both required for the proper encapsulation of eggs from the parasitoid wasp *Leptipolina boulardi*. From these data we conclude that Rac1 induces Bsk activity and stable actin formation for cellular immune activation, leading to sessile hemocyte release and an increase in the number of circulating hemocytes.

Supplementary material available online at
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Key words: Rho GFPases, Cellular immunity, Hemocytes, Parasitization, Actin cytoskeleton

Introduction

The *Drosophila melanogaster* larval cellular immune response involves circulating immune surveillance cells known as hemocytes. In *Drosophila*, larval hemocytes develop in the lymph gland, a hematopoietic organ consisting of multiple pairs of lobes located behind the brain (Meister, 2004). There is also a second sessile hemocyte population just underneath the larval cuticle arranged in a segmental pattern (Goto et al., 2003; Lanot et al., 2001; Zettervall et al., 2004). Based on morphology, three basic types of hemocytes can be identified, plasmatocytes, lamellocytes and crystal cells. The most abundant circulating hemocytes are plasmatocytes, small cells that are involved in phagocytosis and able to produce antimicrobial peptides. The largest and normally least abundant hemocytes are the lamellocytes. They are involved in the encapsulation of invading pathogens and are rarely seen in healthy larvae but become enriched when larvae are parasitized (Carton and Nappi, 1997; Lanot et al., 2001; Sorrentino et al., 2002). Crystal cells secrete components of the phenol oxidase cascade, which is involved in melanization of invading organisms and in wound repair (reviewed in Meister, 2004).

When an invading organism is recognized as foreign, circulating hemocytes should rapidly remove it by phagocytosis and/or encapsulation. This reaction can be observed when the parasitoid wasp *Leptopilina boulardi* lays its eggs in the hemocoel of second-instar *Drosophila* larvae. Parasitization elicits a strong cellular response, inducing the release of hemocytes from the lymph gland (Lanot et al., 2001) and also the sessile population (Zettervall et al., 2004). Furthermore, it causes the differentiation of numerous lamellocytes (Carton and Nappi, 1997; Meister, 2004; Meister and Lagueux, 2003). Once a wasp egg is recognized, capsule formation ensues. This requires circulating plasmatocytes to change from non-adhesive to adhesive, enabling them to adhere to the invader. After the plasmatocytes attach and spread around the chorion of the wasp egg they form septate junctions. This effectively separates the wasp egg from the larval hemocoel. The last phases of capsule formation include lamellocyte adherence, and melanization due to crystal cell degranulation (Russo et al., 1996).

From these encapsulation events it is obvious that adhesion and cell shape change are an essential part of the cellular immune response against parasitoid wasp eggs. Rac GTPases are known to regulate the cytoskeletal rearrangements and adhesions necessary for cell-shape change and migration (reviewed in Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004). Cell migration can be subdivided into a series of sequential events, including lamellipodium extension, formation of new adhesions, cell-body contraction and tail detachment (reviewed in Ridley, 2001; Small et al., 2002). Lamellipodia formation requires the polymerization of actin branches, leading to the extension of a lamella in the direction of migration (reviewed in Ridley, 2001). Branched actin polymerization during lamellipodium extension is under the

control of Rac GTPases (Miki et al., 1998), whereas the direction of migration is controlled by the Rho family member Cdc42 (Allen et al., 1998). After the lamella is extended there is adhesion of the leading edge to the substrate. This requires the interaction of adhesion receptors with the extracellular matrix outside of the cell, and the actin cytoskeleton inside of the cell (Hotchin and Hall, 1995). These initial adhesions formed at the leading edge are known as focal contacts. It is believed that Rac plays an active part in regulating focal contact formation (Nobes and Hall, 1995). Once these interactions form, Rho activity leads to the maturation of focal contacts into focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). Focal adhesions allow for the force that is created by cellular contraction of the actin cytoskeleton to be converted into cell movement. During the final stage, the trailing edge of the migrating cell is released from the extracellular matrix and retracts towards the front of the cell. For this retraction to occur the focal adhesions must be turned over and contraction of the cytoskeleton by actomyosin can then begin to pull the rear of the cell forward (reviewed in Ridley, 2001).

The *Drosophila* genome encodes two Rac GTPases (Rac1 and Rac2). A third homolog Mig-2-like (Mtl) has similarity to both Rac and Cdc42 GTPases, but signals more like Rac GTPases (Hakeda-Suzuki et al., 2002; Newsome et al., 2000). In *Drosophila*, Rac GTPases are involved in the cell movements necessary for proper development, and during embryogenesis the three Racs are redundant (Hakeda-Suzuki et al., 2002; Ng et al., 2002). Paladi and Tepass reported that Rac1 and Rac2 are necessary in a redundant fashion for the migration of *Drosophila* embryonic hemocytes (Paladi and Tepass, 2004), and Stramer et al. showed that Rac activity is necessary for hemocyte migration into embryonic wounds (Stramer et al., 2005). All these observations suggest that Rac GTPases play a central role in cell migration in the *Drosophila* embryo. In *Drosophila* larvae, Rac1 and Rac2 are also involved in regulating hemocyte activation. The overexpression of wild-type Rac1 in larval hemocytes significantly increases the number of circulating plasmatocytes and lamellocytes (Zettervall et al., 2004). Rac2 has a specific role in cellular spreading during the encapsulation process of invading parasitoid eggs from the wasp *L. boulardi* (Williams et al., 2005).

We report here that Rac1 requires the *Drosophila* Jun kinase basket (Bsk) as well as stable actin formation to recruit the sessile hemocyte population and increase the number of circulating hemocytes. Furthermore, we show that Rac1 and Bsk are involved in the regulation of cellular adhesions in activated hemocytes. We also show that Rac1 and Bsk are both required for the proper encapsulation of eggs from the parasitoid wasp *L. boulardi*.

Results

Rac1 GTPase activates two pathways to induce sessile hemocyte release

The overexpression of wild-type Rac1 in larval hemocytes disrupts the sessile hemocyte population and significantly increases the number of circulating hemocytes (Zettervall et al., 2004). It is known from other studies that Rac1 activation causes the dissociation of inhibitory proteins from the WASp family protein SCAR. SCAR can then interact with the Arp2/3 complex and stimulate the branched actin formation necessary for lamellipodia formation (Kunda et al., 2003; Rogers et al., 2003). Rac1 also regulates a MAP kinase cascade, ultimately leading to Jun-kinase activation (reviewed in Gallo and Johnson, 2002; Huang et al., 2004). One mutant of *Drosophila* Rac1, $Rac1^{F37A}$, can activate Jun kinase but is defective in inducing lamellipodium extension (Joneson et al., 1996; Ng et al., 2002). A second mutant, $Rac1^{Y40C}$, can induce lamellipodia formation but cannot activate Jun kinase (Joneson et al., 1996; Ng et al., 2002). We decided to use these various alleles to elucidate what is required downstream of Rac1 to disrupt the sessile hemocyte segmental banding pattern, and increase the number of circulating hemocytes.

To study the effect of Rac1 signalling on the sessile hemocyte population various *UAS-Rac1* transgenic flies were crossed to *Hemese-GAL4*, *UAS-GFPnls* driver flies (hereafter called *He-Gal4*). In third-instar control larvae, segmentally arranged hemocytes were observed just underneath the cuticle (Fig. 1A). Overexpression of wild-type Rac1 GTPase specifically in hemocytes disrupted this segmental banding pattern (Fig. 1B). The overexpression of the Rac1-effector-loop mutants $Rac1^{F37A}$ or $Rac1^{Y40C}$ in hemocytes had little effect on the sessile hemocyte population (Fig. 1C,D). Using the *He-Gal4* driver we coexpressed $Rac1^{F37A}$ and $Rac1^{Y40C}$ in hemocytes and found that the phenotype was similar to that caused by Rac1 overexpression: the sessile hemocyte-banding pattern was disrupted (Fig. 1E). The expression of dominant-negative Rac1 ($Rac1^{N17}$) in hemocytes did not disrupt the sessile hemocyte-banding pattern (Fig. 1F). Examination of protein expression levels in hemocytes showed all the transgenic constructs were overexpressed when crossed with *He-Gal4* and produced stable proteins (supplementary material Fig. S1).

It has previously been reported that wild-type Rac1, when overexpressed in hemocytes, causes an increase in the number of circulating plasmatocytes; approximately three times more plasmatocytes were in circulation than in equally aged control larvae (Zettervall et al., 2004). There was also a significant increase in the number of circulating lamellocytes (Fig. 1G). No increase in circulating hemocytes was observed when either of the Rac1-effector-loop mutants was overexpressed. When the Rac1-effector-loop mutants were expressed in the same larvae, there was a significant increase in the number of circulating plasmatocytes and also an increased number of lamellocytes (Fig. 1G). We conclude that Rac1 must activate two pathways to recruit the sessile hemocyte population, increase the number of circulating plasmatocytes and induce lamellocyte formation.

Rac1 requires two pathways to fully activate circulating hemocytes

To examine hemocyte morphology we bled early wandering third-instar larvae and stained the hemocytes with TRITC-phalloidin to visualize their actin cytoskeleton. Hemocytes from control larvae were round in appearance with little F-actin at the plasma membrane (Fig. 2A). Overexpression of Rac1 in hemocytes induced plasma membrane ruffling, with more F-actin visible at the cell periphery (Fig. 2B). When compared with control hemocytes, overexpression of wild-type Rac1 induced a 15-fold increase in the amount of cellular F-actin (Fig. 2G). Hemocytes expressing $Rac1^{F37A}$ had thick actin cables running from the center to the periphery of the cell (Fig. 2C). When these

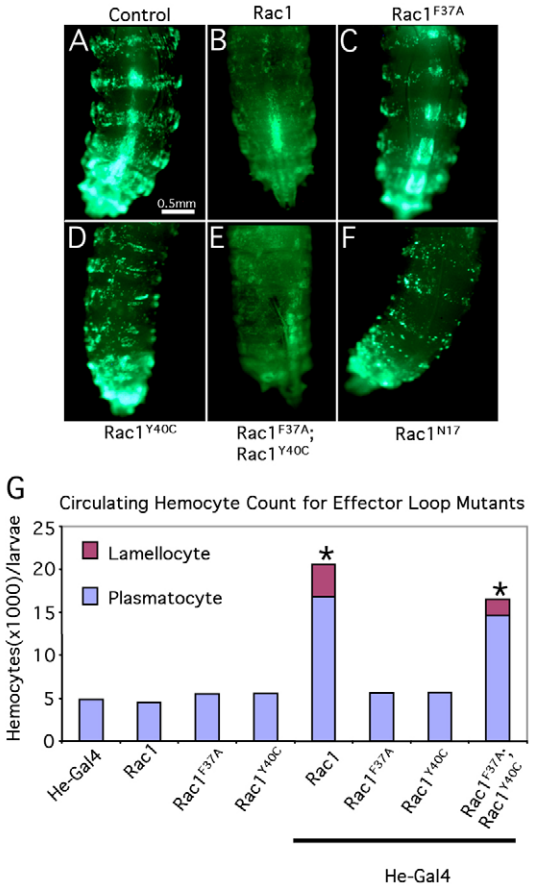

Fig. 1. Rac1 effector loop mutants fail to disrupt the sessile hemocyte population. (A-F) GFP expression in sessile hemocytes of control larvae and larvae expressing various alleles of Rac1 (A) *He-Gal4* (B) *UAS-Rac1*; *He-Gal4* (C) *UAS-Rac1^{F37A}; He-Gal4* (D) *UAS-Rac1^{Y40C}; He-Gal4* (E) *UAS-Rac1^{F37A}; UAS-Rac1^{Y40C}; He-Gal4* (F) *UAS-Rac1^{N17}/He-Gal4*. (G) Hemocyte counts after overexpression of various Rac1 alleles. *He-Gal4* was crossed with the different Rac1 alleles. Hemocytes were counted from at least 15 individual larvae. *, significant difference (Student's *t*-test, $P<0.01$) compared with the parental *UAS* and *He-Gal4* strains.

hemocytes were co-stained with anti-phosphorylated-tyrosine antibody, the staining was localized to the tips of the actin cables, indicating that the structures could be stress fibers (Zimerman et al., 2004). Rac1^{F37A} overexpression induced an approximately fivefold increase in cellular F-actin (Fig. 2G). Hemocytes expressing Rac1^{Y40C} had ruffled membranes, although not to the same extent as Rac1 wild-type cells, and different amounts of actin accumulated at the periphery of the cell (Fig. 2D). Similar to Rac1^{F37A}, Rac1^{Y40C} induced an approximately fivefold increase in F-actin (Fig. 2G). Hemocytes bled from larvae coexpressing Rac1^{F37A} and Rac1^{Y40C} looked similar to larvae that overexpressed wild-type Rac1, having an increased F-actin accumulation at the cell periphery (Fig. 2E). Similar to wild-type Rac1 overexpression, hemocytes coexpressing Rac1^{F37A} and Rac1^{Y40C} had a 12-fold increase in F-actin (Fig. 2G). This shows that Rac1 must activate two pathways for stable formation of lamellipodia.

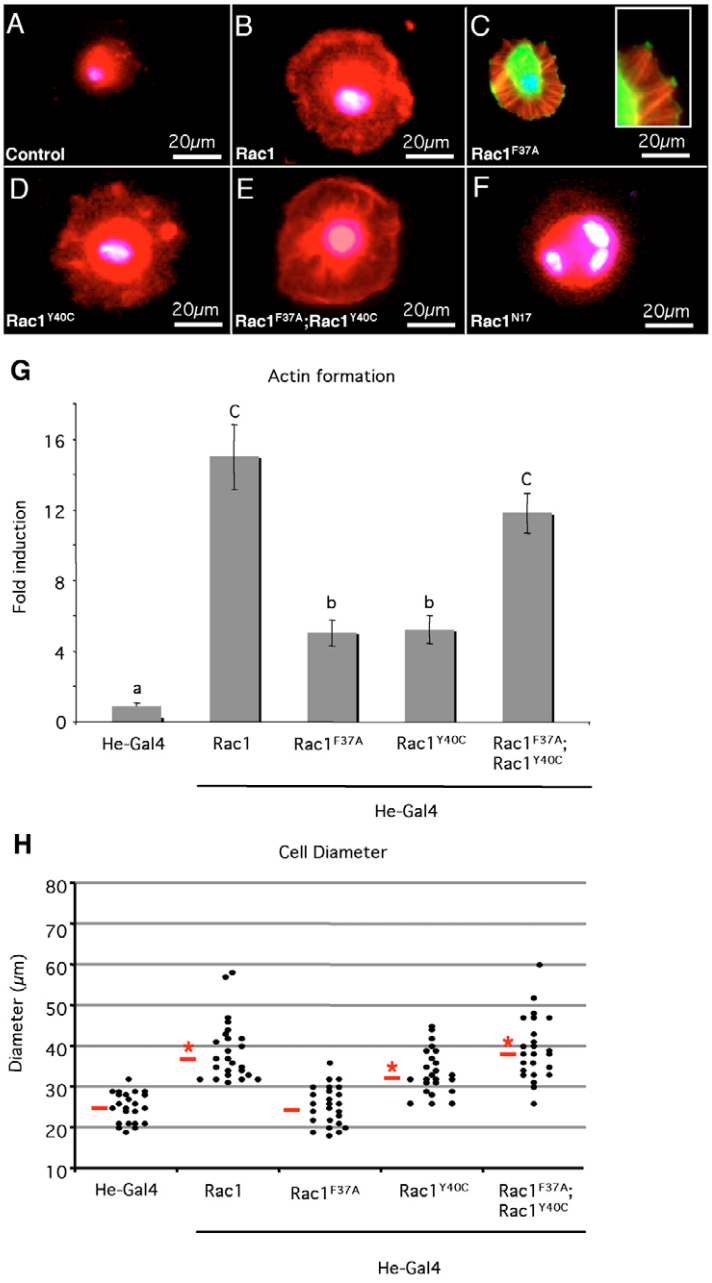

Fig. 2. Rac1 must activate two pathways to induce hemocytes activation. (A-F) Hemocyte actin cytoskeleton was visualized using TRITC-phalloidin (red), the nucleus was stained with DAPI (blue) (A) *He-Gal4* (B) *UAS-Rac1; He-Gal4* (C) *UAS-Rac1^{F37A}*; *He-Gal4*, hemocytes were also stained with anti-phosphorylated-tyrosine antibody (green) (D) *UAS-Rac1^{Y40C}*; *He-Gal4* (E) *UAS-Rac1^{F37A}*; *UAS-Rac1^{Y40C}*; *He-Gal4* (F) *UAS-Rac1^{N17}/He-Gal4*. (G) F-actin expression levels of the various Rac1 alleles. *He-Gal4* was crossed with different Rac1 alleles and hemocytes were bled from wandering third-instar larvae. The hemocytes were stained with TRITC-phalloidin. Imagetrak was used to measure fluorescence intensity of at least 100 hemocytes from three different larvae. Different letters indicate similar groups (i.e. 'a' is significantly different from 'b' or 'c' and so on; Student's *t*-test, $P<0.01$). (H) Determination of plasmatocyte diameter. The cell diameter of plasmatocytes from the various genotypes was measured, as described in Materials and Methods, and the diameter (μm) for 25 hemocytes was plotted. *, significant difference (Student's *t*-test, $P<0.01$) compared with the parental *UAS* and *He-Gal4* strains.

During these experiments it became obvious that the various Rac1 alleles had an effect on cell spreading. Fig. 2H shows that hemocytes that were bled from control larvae have a median diameter of 25 μm. Overexpression of Rac1 in hemocytes significantly increased their median diameter to 37 μm (Fig. 2H). When compared with Rac1-overexpressing cells, hemocytes expressing $Rac1^{F37A}$ were small, with a median diameter of just 26 μm (Fig. 2H), whereas $Rac1^{Y40C}$ hemocytes had a median diameter of 32 μm, just slightly smaller than that of cells expressing wild-type Rac1 (Fig. 2H).

Overexpression of dominant-negative Rac1 ($Rac1^{N17}$) in hemocytes resulted in an interesting phenotype. When hemocytes expressing $Rac1^{N17}$ were stained with TRITC-phalloidin, no F-actin was evident at the cell periphery and the cells had a greater diameter than control hemocytes (Fig. 2F). DAPI staining revealed that many of the cells were bi- or multinucleate (Fig. 2F). This might be because Rac1 has a role in hemocyte cytokinesis or because the overexpression of dominant-negative Rac1 interferes with the cytokinesis machinery.

Loss of cofilin rescues $Rac1^{Y40C}$ lamellipodia

Although the expression of $Rac1^{Y40C}$ in hemocytes can induce the formation of lamellipodia, it was not as extensive as in hemocytes overexpressing wild-type Rac1 (compare Fig. 3A with B). It was also apparent from phalloidin staining that the amount of F-actin at the periphery of $Rac1^{Y40C}$-expressing hemocytes was lower than in hemocytes overexpressing wild-type Rac1. This could mean that $Rac1^{Y40C}$ cannot induce the formation of F-actin to the same level as wild-type Rac1, or that it cannot block the breakdown of F-actin by cofilin. Rac1 signals upstream of Lim kinase to inhibit cofilin, this inhibition leads to the formation of stable actin (Chen et al., 2005; Raymond et al., 2004). Mammalian cell studies have shown that $Rac1^{V12H40}$ (a mutant similar to $Rac1^{Y40C}$) cannot activate this pathway (Joneson et al., 1996); therefore, we decided to test the latter of these alternatives. To express $Rac1^{Y40C}$ in hemocytes that lack one copy of the *Drosophila* cofilin gene twinstar *(tsr)*, we crossed *UAS-*$Rac1^{Y40C}$*; He-Gal4* with *tsr*^{k05633}*/Cyo, Kr-Gal4, UAS-GFP*. Hemocytes bled from *tsr*^{k05633}*/Cyo, Kr-*

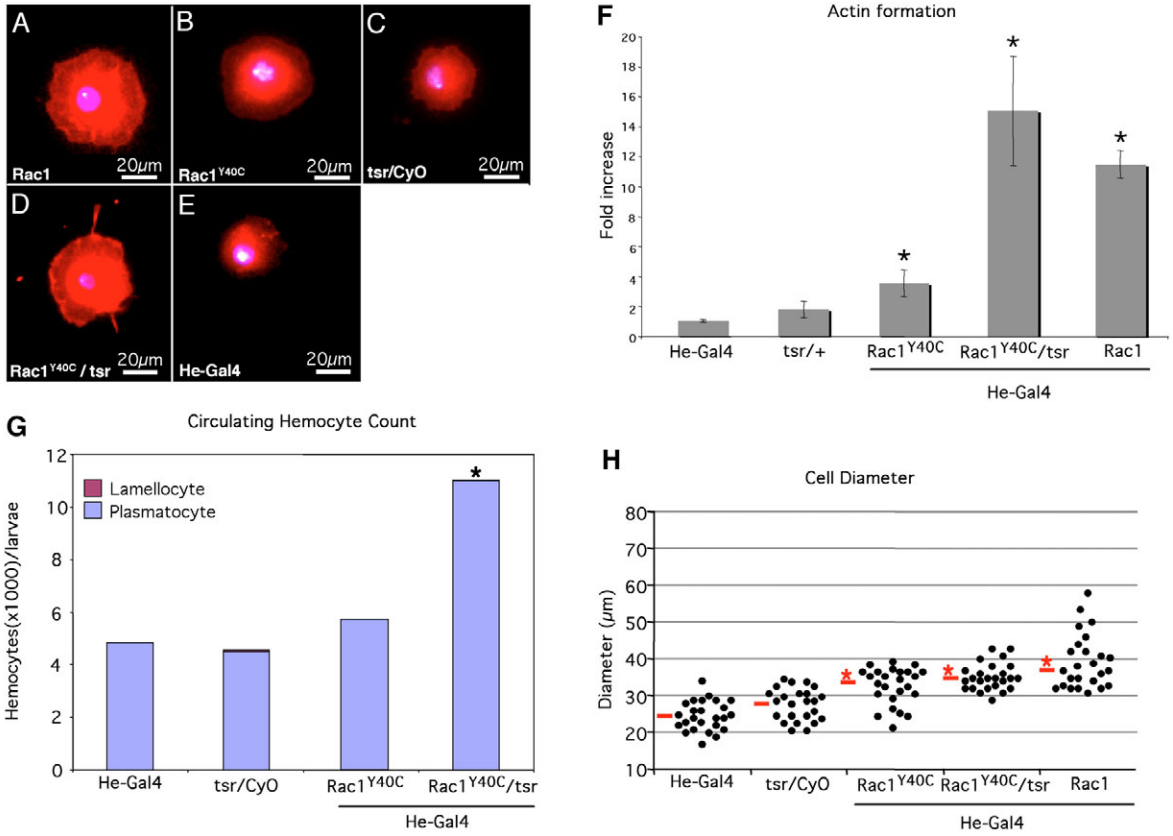

Fig. 3. Reducing the amounts of cofilin partially rescues $Rac1^{Y40C}$. (A-E) Hemocytes were bled from early third-instar larvae and stained with TRITC-phalloidin (red), the nucleus was stained with DAPI (blue). (A) *UAS-Rac1;He-Gal4* (B) *UAS-*$Rac1^{Y40C}$; *He-Gal4* (C) *y[1] w[67c23]; P{w[+mC]=lacW}tsr[k05633]/CyO, Kr-Gal4, UAS-GFP* (D) *UAS-*$Rac1^{Y40C}$*/P{w[+mC]=lacW}tsr[k05633]*; *He-Gal4* (E) *He-Gal4*. (F) F-actin expression levels of different Rac1 alleles crossed to *He-Gal4* and *y[1] w[67c23]; P{w[+mC]=lacW}tsr[k05633]/CyO, Kr-Gal4, UAS-GFP*. Hemocytes were bled from wandering third-instar larvae and stained with TRITC-phalloidin. Imagetrak was used to measure fluorescence intensity of at least 100 hemocytes from three different larvae. *, significant difference (Student's *t*-test, $P<0.01$) compared with the parental *UAS* and *He-Gal4* strains. (G) Hemocyte counts after overexpression of various *UAS* alleles. Hemocytes were counted from at least 15 individual larvae. *, significant difference (Student's *t*-test, $P<0.01$) compared with the parental *UAS* and *He-Gal4* strains. (H) Determination of plasmatocyte size. The cell diameter of plasmatocytes from the various genotypes was measured, as described in Materials and Methods, and the diameter (μm) for 25 hemocytes was plotted.

Gal4, UAS-GFP larvae showed an increase in F-actin at the cell periphery when compared with hemocytes from control larvae (compare Fig. 3C with E). There was an approximately twofold increase in the total amount of cellular F-actin when compared with control hemocytes (Fig. 3F). When $Rac1^{Y40C}$ was expressed in cells with reduced levels of Tsr, the amount of membrane ruffling was similar to that seen in Rac1-overexpressing hemocytes, and there was an approximately 16-fold increase in the amount of F-actin (Fig. 3D,F). Reducing the levels of Tsr in $Rac1^{Y40C}$ plasmatocytes did not significantly change the cell diameter (Fig. 3H). From this, we conclude that Rac1 must inhibit cofilin, as well as induce formation of new F-actin to form stable lamellipodia.

During these experiments, we noticed that the *$Rac1^{Y40C}$/tsr; He-Gal4* larvae seemed to have an increased number of circulating hemocytes. As seen before, when $Rac1^{Y40C}$ was overexpressed in hemocytes, no increase in either circulating plasmatocytes or activated lamellocytes was observed (Fig. 3G). *tsr^{k05633}/Cyo, Kr-Gal4, UAS-GFP* larvae also showed no increase in the numbers of plasmatocytes or lamellocytes. However, when $Rac1^{Y40C}$ was overexpressed and one copy of *tsr* was removed, there was a significant increase in the number of circulating plasmatocytes (Fig. 3G). The sessile hemocyte-banding pattern was also disrupted (data not shown). Thus, stable actin formation is sufficient to significantly increase the number of circulating plasmatocytes, but not sufficient for lamellocyte formation.

Effects of Rac1-effector-loop mutants on Bsk activation

To investigate further the activities of the Rac1-effector-loop mutants in hemocytes, we examined their ability to activate the *Drosophila* Jun N-terminal kinase homolog Bsk. During the final stages of larval development, just before pupation, Bsk becomes phosphorylated in circulating hemocytes, indicating a higher level of activiated Bsk (data not shown). To avoid this high level of endogenous activated Bsk, we bled hemocytes from early third-instar larvae. When hemocytes were bled from control larvae (*He-Gal4* or CantonS) and stained with an antibody that recognizes activated Bsk, staining was observed at the periphery of the cell and in the nucleus (Fig. 4A). There was very little active Bsk evident in the cytoplasm of control hemocytes between the nucleus and cell periphery. Overexpression of wild-type Bsk in hemocytes produced high levels of activiated Bsk throughout the cell, but had no obvious effect on hemocyte morphology, the sessile hemocyte population, or the number of circulating hemocytes (Fig. 4B and data not shown). Overexpression of wild-type Rac1 in hemocytes induced a fourfold increase in the amount of active Bsk (Fig. 4C,F). This activity was observed in the nucleus, as well as in puncta distributed throughout the cell (Fig. 4C). Hemocytes expressing the Rac1-effector-loop mutant $Rac1^{Y40C}$, which cannot activate Jun kinase, looked very similar to control hemocytes (compare Fig. 4D and 4A). When $Rac1^{F37A}$ was overexpressed the cytoplasmic Bsk activity was not localized, but diffuse throughout the cytoplasm (Fig. 4E), unlike in hemocytes expressing wild-type Rac1. Expression of $Rac1^{F37A}$ in hemocytes induced activated Bsk 2.5-fold (Fig. 4F). From these results, we conclude that $Rac1^{F37A}$ can activate Bsk signalling, but this activation is not sufficient to properly localize Bsk.

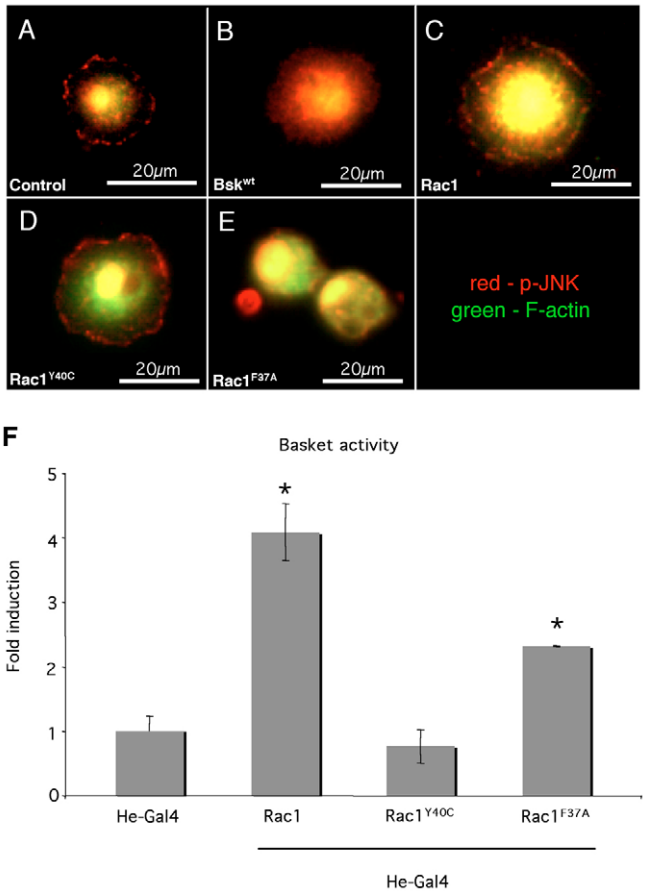

Fig. 4. Bsk activation in hemocytes. Hemocytes were recovered from early third-instar larvae and stained for Bsk activation using anti-phosphorylated-JNK antibody (red). Cells were counter-stained with FITC-phalloidin to visualize their actin cytoskeleton (green). In the merged pictures, overlap of the two stainings is yellow. (A) *He-Gal4* (B) *UAS-Bsk^{A-Y}; He-Gal4* (C) *UAS-Rac1*; *He-Gal4* (D) *UAS-$Rac1^{Y40C}$*; *He-Gal4* (E) *UAS-$Rac1^{F37A}$*; *He-Gal4*. (F) Quantification of Bsk activity. Imagetrak was used to measure fluorescence intensity of at least 100 hemocytes from three different larvae. *, significant difference (Student's *t*-test, $P<0.01$) compared with the parental *UAS* and *He-Gal4* strains.

Bsk signals downstream of Rac1

We next wanted to determine whether Bsk is needed downstream of Rac1 to increase the number of circulating hemocytes. We therefore expressed *UAS-BskIR*, which expresses a Bsk RNAi construct (Ishimaru et al., 2004), together with *UAS-Rac1*. When Bsk signalling was inhibited it completely blocked the release of the sessile hemocyte population, as well as the concurrent increase in circulating plasmatocytes and appearance of lamellocytes induced by Rac1 overexpression (Fig. 5E,G). This suggests that, downstream of Rac1, Bsk is necessary to recruit the sessile hemocyte population, increase the number of circulating plasmatocytes and induce lamellocyte formation.

As described above, Rac1 induces lamellipodia and an increase in cell diameter. This is particularly obvious when Rac1-overexpressing cells are compared with the minority of hemocytes that do not express Bsk RNAi (Fig. 5B, arrow). The loss of Bsk signalling downstream of Rac1 had no effect on

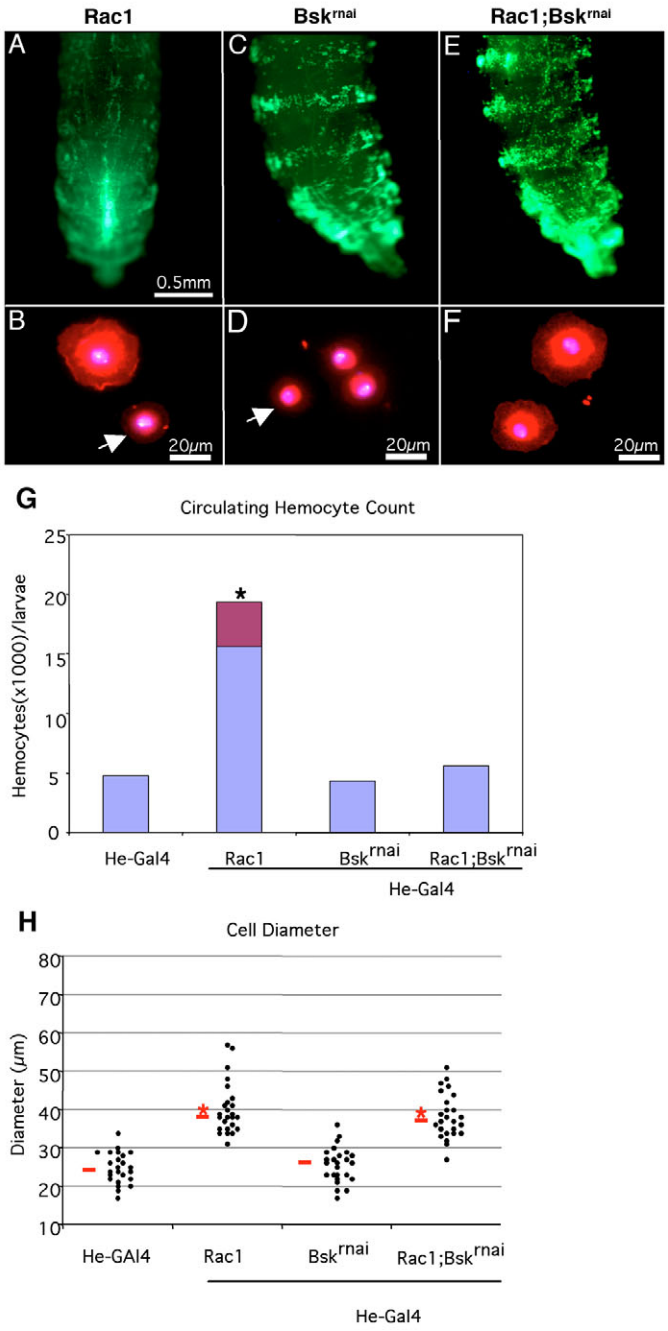

Fig. 5. Bsk is necessary for Rac1-induced increases in circulating hemocytes. (A,C,E) GFP expression in sessile hemocytes larvae expressing (A) *UAS-Rac1*; *He-Gal4* (C) *UAS-BskIR*/*He-Gal4* (E) *UAS-Rac1;UAS-BskIR*/*He-Gal4*. (B,D,F) In respective larvae, hemocyte actin cytoskeleton was visualized with TRITC-phalloidin, the nucleus was stained with DAPI. Arrows indicate cells not expressing the transgene. The He-Gal4 driver flies also contain an UAS-GFPnls transgene. GFP was used to indicate transgene expression. GFP expression is not shown in these figures. (G) Hemocyte counts after overexpression of various *UAS* alleles. Hemocytes were counted from at least 15 individual larvae. (H) Determination of plasmatocyte diameter. The cell diameter of plasmatocytes from the various genotypes was measured on their *x* and *y* axes, the average of 25 hemocytes was plotted in μm. *, significant difference (Student's *t*-test, $P<0.01$) compared with the parental *UAS* and *He-Gal4* strains.

the ability of Rac1 to induce lamellipodia or increase the diameter of hemocytes (Fig. 5F). Expression of Bsk RNAi alone had no obvious effect on the sessile hemocyte population or the formation of F-actin (Fig. 5C,D; arrow indicates a hemocyte not expressing Bsk RNAi). Overexpression of Rac1 in hemocytes increased their diameter significantly, to a median diameter of 37 μm (Fig. 5H). Control hemocytes had a median diameter of 24 μm (Fig. 5H). When Bsk signalling was inhibited downstream of Rac1, the median hemocyte diameter was still 37 μm (Fig. 5H). From these results we conclude that, although Bsk is required to mobilize sessile cells, it is dispensable for Rac1-induced formation of lamellipodia in plasmatocytes.

Rac1 and Bsk regulate hemocyte cellular adhesions

Since lamellocyte formation induced by overexpression of wild-type Rac1 requires Bsk signalling, we decided to see whether Rac1 and Bsk are necessary for lamellocyte formation after parasitization. Control larvae (either Hml^{Δ}-*Gal4* or *UAS-BskIR*), larvae expressing *UAS-BskIR* under the control of Hml^{Δ}-*Gal4* and homozygous $Rac1^{J11}$ loss-of-function larvae were parasitized by the avirulent *L. boulardi* wasp strain G486. We used the Hml^{Δ}-*Gal4* driver for this experiment, because unlike the *He-Gal4* driver, Hml^{Δ}-*Gal4* is constitutively expressed in the lymph gland. The lymph gland is activated by wasp parasitization, thereafter producing and releasing many lamellocytes (Lanot et al., 2001). Forty hours after parasitization, a significant increase in the number of circulating lamellocytes was seen in all cases (Fig. 6A). From this, we conclude that Rac1 and Bsk are not necessary for the formation of lamellocytes induced by wasp parasitization.

Jun kinase is known to be involved in regulating focal adhesions in vertebrate cell lines (Huang et al., 2003). We bled hemocytes 40 hours after parasitization to examine whether Rac1 and Bsk are involved in regulating focal adhesions in hemocytes. The bled hemocytes were stained with an antibody against phosphorylated focal adhesion kinase (FAK) (anti-phosphorylated-FAK), and anti-phosphorylated-tyrosine antibody. Since many of the proteins involved in the maintenance of cellular adhesions are phosphorylated on tyrosines including FAK, this allowed us to visualize focal adhesions (reviewed in Playford and Schaller, 2004). In lamellocytes from parasitized control larvae, no colocalization of phosphorylated FAK, phosphorylated tyrosine and F-actin was ever observed (Fig. 6B). In lamellocytes that lack either active Rac1 or active Bsk, phosphorylated FAK and phosphorylated tyrosine were co-localized at large placodes (Fig. 6B, see arrows), which coincided with higher levels of F-actin (Fig. 6B). Similar results were obtained when plasmatocytes were stained (supplementary material Fig. S2). These results suggest that Rac1 and Bsk regulate the formation of these actin- and FAK-rich placodes in activated lamellocytes after parasitization.

Usually, the darkened cellular capsule surrounding a parasitoid wasp egg is easily visible in the hemocoel of wild-type *Drosophila* larvae 30 to 40 hours after parasitization by the avirulent *L. boulardi* wasp strain G486. Yet, while doing these experiments, we noticed that homozygous $Rac1^{J11}$ loss-of-function mutants and Hml^{Δ}*;BskIR* larvae failed to properly melanize the wasp egg. We used this finding as the basis for a wasp encapsulation assay to test how *Rac1* loss-of-function

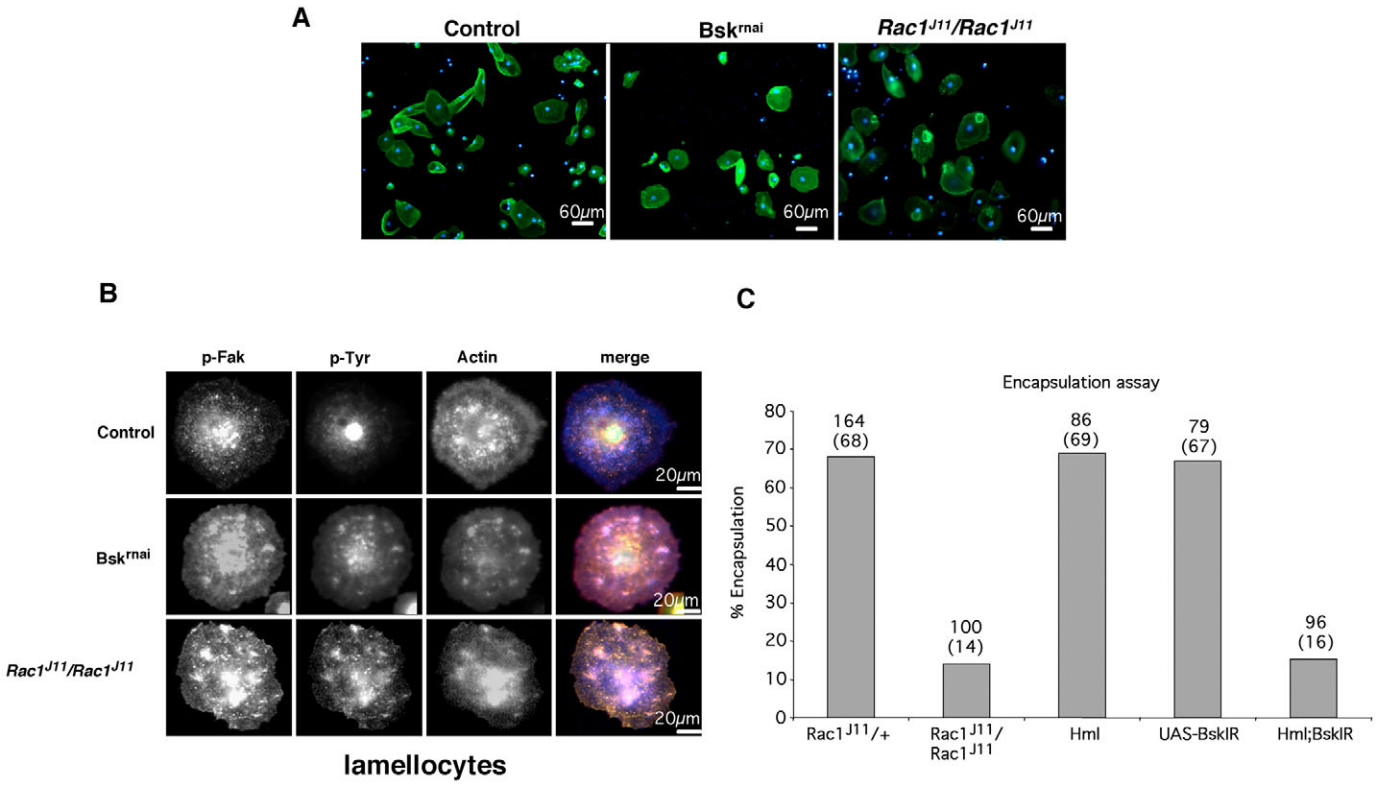

Fig. 6. Loss of Rac1 or Bsk does not block lamellocyte activation after parasitization. Hemocytes were recovered from larvae 40 hours after parasitization by the parasitoid *L. boulardi* G486. (A) Lamellocytes were recovered from parasitized *Hml*$^{\Delta}$*-Gal4* control, *UAS-BskIR/Hml*$^{\Delta}$*-Gal4* or, homogygous *Rac1*^{J11} loss-of-function larvae and stained with the L1 lamellocyte-specific antibody (green), the nucleus was stained with DAPI (blue). (B) Lamellocytes recovered from parasitized *Hml*$^{\Delta}$*-Gal4* control, *UAS-BskIR/Hml*$^{\Delta}$*-Gal4*, or homogygous *Rac1*^{J11} loss-of-function larvae and stained with anti-phosphorylated-FAK antibody (red), anti-phosphorylated-tyrosine antibody (green) and Alexa-Fluor-350-phalloidin (blue). In the merged pictures, overlap of the three stainings is light violet (arrows). (C) Encapsulation of wasp eggs in parasitized *Hml*$^{\Delta}$*-Gal4* control *UAS-BskIR/Hml*$^{\Delta}$*-Gal4* larvae or homogygous *Rac1*^{J11} loss-of-function larvae. Numerical values for proper encapsulation percentages [(Number of properly melanized wasp eggs/number of parasitized larvae) × 100] are presented above each bar. Numbers indicate the number of wasp-parasitized larvae. Numbers in parentheses indicate percentage of total larvae with a properly melanized wasp egg.

mutants as well as the lack of active Bsk effects the cellular immune reaction (Sorrentino et al., 2002). In *Rac1*^{J11}*/TM6,Tb* control larvae, 40 to 42 hours after parasitization, 68% of the wasp eggs were correctly encapsulated. In *Rac1*^{J11} homozygotes, however, the rate of proper encapsulation was 14% (Fig. 6C), and *Hml*$^{\Delta}$*;BskIR* larvae properly encapsulated only 16% of the wasp eggs. In *Hml*$^{\Delta}$*-Gal4* or *UAS-BskIR* control larvae proper encapsulation was observed 69% or 67%, respectively (Fig. 6C). From this, we conclude that both Rac1 and Bsk are necessary for proper capsule formation in response to eggs from the parasitoid *L. boulardi*.

Bsk activation after parasitization is partially controlled by Rac1

Plasmatocytes bled from parasitized control larvae 40 hours post-parasitization had an approximately sevenfold increase of active Bsk compared with plasmatocytes of non-parasitized control larvae (Fig. 7A,B). Similar to wild-type Rac1-overexpressing hemocytes (see Fig. 4C), active Bsk was in the nucleus and in puncta throughout the cytoplasm (Fig. 7A). Plasmatocytes bled from parasitized homozygous *Rac1*^{J11} larvae 40 hours post-parasitization had partially reduced induction of Bsk activity (Fig. 7A,B). Interestingly, most of the active Bsk in hemocytes from Rac1^{J11} larvae was located in the nucleus (Fig. 7A). Unlike controls, there was very little active Bsk observed in the cytoplasm. From these results, we conclude that Rac1 is necessary for some, but not all, of the Bsk activation seen after parasitization.

Discussion

Taken together, we found that Rac1 GTPase requires activation of Bsk, as well as formation of stable actin to induce the *Drosophila* larval cellular immune response (Fig. 8). The most compelling evidence for a role of Rac1 and Bsk in the cellular immune response is the lack of encapsulation in response to the parasitoid *L. boulardi*. Recently, Labrosse et al. reported that one of the genes found in the polydnaviruses injected when the parasitoid wasp *L. boulardi* parasitizes *Drosophila*, encodes a RhoGAP (Labrosse et al., 2005a). Interestingly, this RhoGAP is more similar to Rac-specific GAPs. The group went on to show that this RhoGAP inhibited lamellocyte-production as well as -function (Labrosse et al., 2005b). Also, loss of Rac2 activity in larval hemocytes totally inhibits proper encapsulation of wasp eggs (Williams et al., 2005). This is evidence that Rho family GTPases are central players in the regulation of *Drosophila* larval cellular immune activation.

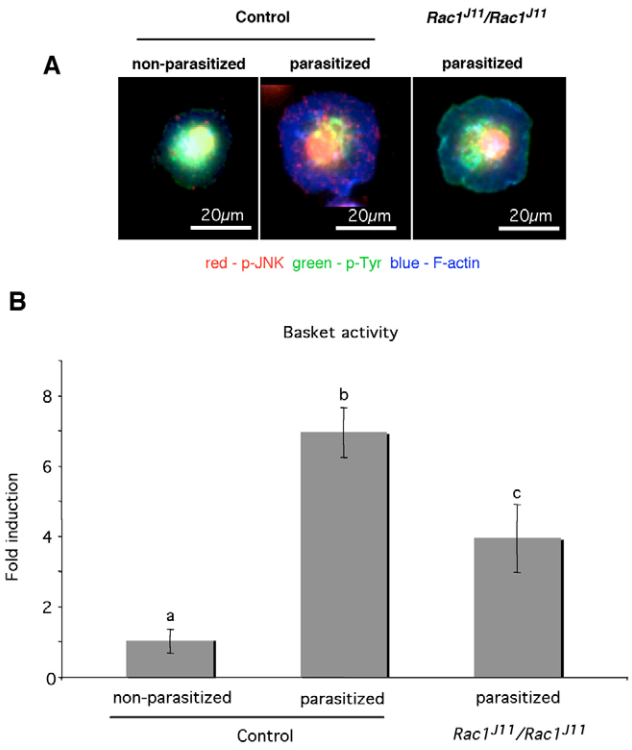

Fig. 7. Bsk activation in parasitized hemocytes. (A) Hemocytes were recovered from non-parasitized and parasitized control (*Rac1*^{J11}*/TM6b,Tb*) or homogygous *Rac1*^{J11} loss-of-function third-instar larvae and stained for Bsk activation with anti-phosphorylated-JNK antibody (red), anti-phosphorylated-tyrosine antibody (green), and Alexa-Fluor-350-phalloidin (blue). (B) Quantifying Bsk activity. Imagetrak was used to measure fluorescence intensity of at least 100 hemocytes from three different larvae. Different letters indicate similar groups (i.e. 'a' is significantly different from 'b' or 'c' and so on; Student's *t*-test, $P<0.01$).

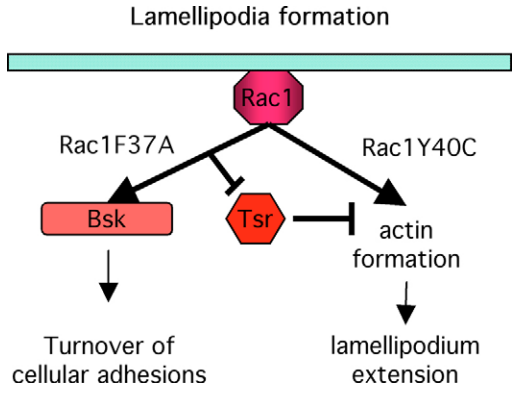

Fig. 8. Schematic diagram showing Rac1 involvement in lamellipodia formation.

Rac1 induced increase in circulating hemocyte numbers.

The expression of either Ras85D or Egfr in hemocytes leads to an approximately 60-fold increase in the number of circulating plasmatocytes compared with control larvae (Asha et al., 2003; Zettervall et al., 2004). The large increase induced by the EGF receptor pathway can only be explained by increased hemocyte proliferation. Overexpression of wild-type Rac1 increases the number of circulating plasmatocytes only 3-fold (Zettervall et al., 2004) (this study). This increase might be explained by the release of the sessile hemocyte population, although we cannot rule out the possibility that proliferation is also involved.

At present, it is not known how sessile hemocytes are maintained in a segmental pattern underneath the larval epidermis, or what the mechanism is that induces their release into circulation. We used two Rac1-effector-loop mutants to elucidate what is required downstream of Rac1 to disrupt the sessile hemocyte segmental banding pattern. Although Rac1^{F37A} activates Jun kinase and Rac1^{Y40C} induces the formation of branched-actin leading to lamellipodia (Joneson et al., 1996; Ng et al., 2002), neither mutant on its own is sufficient to cause sessile hemocyte release or an increase in the number of circulating hemocytes. We speculate that Rac1^{Y40C} can induce the formation of F-actin but not the inhibition of cofilin. Endogenous Rac1 acts upstream of Lim kinase to inhibit cofilin; this inhibition leads to formation of stable F-actin (Chen et al., 2005; Raymond et al., 2004). Although our study is not conclusive, it is possible that, as well as inducing actin formation, Rac1 overexpression in hemocytes inhibits cofilin. This inhibition leads to formation of stable F-actin and might be sufficient for sessile hemocyte release. This is evident when Rac1^{Y40C} is overexpressed and one copy of the *Drosophila* cofilin *tsr* gene is removed. The sessile hemocytes are disrupted and a significant increase in circulating plasmatocytes is observed. Interestingly, this is not sufficient to increase the number of circulating lamellocytes; possibly because of a need for increased Bsk activity or some other, as yet unknown, mechanism downstream of Rac1 to form lamellocytes.

The *Drosophila* Jun kinase Bsk is necessary downstream of Rac1 for sessile hemocyte release, as well as for the formation of lamellocytes. When a Bsk RNAi construct is co-expressed with Rac1 in hemocytes, release of the sessile population is blocked. There is also no concurrent increase in circulating plasmatocytes or the formation of lamellocytes. This means that Bsk is required downstream of Rac1 to disrupt the sessile hemocyte banding pattern. It could also mean that Bsk is required for Rac1-induced formation of lamellocytes. Another intriguing possibility is that the formation of lamellocytes is a secondary event that initially requires sessile hemocytes to be released into circulation. However, as mentioned before, release is not sufficient to induce the formation of lamellocytes.

Rac1 and Bsk regulate actin- and FAK-rich placodes

In vertebrate cell lines it has been shown that Jun kinase phosphorylation of Paxillin is necessary for focal adhesion turnover (Huang et al., 2003) and, in *Drosophila*, Paxillin inhibits Rho function and enhances Rac activation, leading to the inhibition of focal adhesions (Chen et al., 2005). This is evidence of the involvement of Rac1 and Jun kinase in focal adhesion turnover. We found further evidence for Rac1 and Bsk involvement in the regulation of focal-adhesion-like actin- and FAK-rich placodes. We call them focal-adhesion-like placodes because they lack the stress fibers reminiscent of true focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992). When loss-of-function Rac1 larvae or larvae expressing Bsk RNAi in hemocytes were parasitized by

L. boulardi, they formed large plaques of phosphorylated FAK and phosphorylated tyrosine that coincided with an increase of F-actin, which is in contrast to wild-type lamellocytes, This is evidence that in the *Drosophila* cellular immune response against parasitization, Rac1 and Bsk are necessary to regulate focal-adhesion-like placodes. It has not been reported whether lamellocytes can migrate, so at this time the purpose of these actin- and FAK-rich placodes is not known.

Concluding remark

Finally, we must caution that some, or possibly all, of the phenotypes seen when the various Rac1 alleles are overexpressed, might be due to an interference with Rac2. Whereas initial experiments expressing dominant-negative Rac1 gave strong embryonic phenotypes (Harden et al., 1995; Glise and Noselli, 1997), more recent studies using Rac1 and Rac2 loss-of-function alleles showed that these two genes are redundant during embryogenesis (Hakeda-Suzuki et al., 2002; Ng et al., 2002). This is evidence that overexpression of dominant-negative Rac1 can block not only Rac1 signalling, but Rac2 signalling as well. However, we showed that Rac2 has a specific role in cellular spreading during the encapsulation process of invading parasitoid eggs from the wasp *L. boulardi* (Williams et al., 2005) and here we show that Rac1 mutants also fail to properly encapsulate wasp eggs. This is evidence that the Rac GTPases are not redundant during the larval immune response against the parasitoid *L. boulardi*.

Materials and Methods

Insects

Drosophila strains, unless otherwise mentioned, were obtained from the Bloomington Stock Center, and the references are given in Flybase (http://fbserver.gen.cam.ac.uk:7081). *UAS-BskIR* RNAi flies were provided by Ryu Ueda (Ishimaru et al., 2004). *Hemolectin$^{\Delta}$-Gal4, 2X UAS-eGFP* was provided by Sergey Sinenko (Sinenko et al., 2004). Flies were kept on a standard mashed-potato diet at 21-25°C. Stocks crossed with Gal4 driver flies and the uncrossed control flies were raised at 29°C. The G486 strain of *Leptopilina boulardi* (Dupas et al., 1998) was bred on a CantonS stock of *Drosophila melanogaster* at room temperature using a standard medium. Adult wasps were maintained at room temperature on apple-juice plates.

Immunofluorescence

For all antibody stainings hemocytes, 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 hour. Staining and analysis were done according to Zettervall et al. (Zettervall et al., 2004). The lamellocyte monoclonal antibody L1a was used undiluted (Kurucz et al., 2003). The polyclonal anti-active-Jun-kinase antibody (Promega) and the monoclonal anti-phosphorylated-tyrosine antibody (Cell Signaling Technology) were diluted 1:500 in 3% bovine serum albumin (BSA)-PBS. The polyclonal anti-phosphorylated-FAK^{Y397} (Biosource) and the monoclonal anti-Myc (Sigma) antibodies were diluted 1:1000 in 3% BSA-PBS. Double-staining was carried out as stated previously, except that after application of the secondary antibody, cells were washed three times in 1× PBS, before being fixed for 5 minutes in 3.7% paraformaldehyde-PBS. After this, cells were washed three more times with 1× PBS, then phalloidin-stained and washed, and analysed as in Zettervall et al. (Zettervall et al., 2004). FITC-phalloidin (Sigma) was diluted to a final concentration of 0.10 μg/μl, Alexa-Fluor-350-phalloidin (Molecular Probes) was diluted to a final concentration of 0.20 μg/μl in 1× PBS.

For F-actin visualization alone, hemocytes were bled from larvae into 20 μl of PBS and allowed to attach to a glass slide for 1 hour at room temperature. The cells were then fixed for 5 minutes with 3.7% paraformaldehyde-PBS, before being washed once for 5 minutes with PBS, followed by a 5-minute wash with PBST (PBS containing 0.1% of Triton X-100) and a final 5-minute wash with PBS. The cells were then stained for 40 minutes at room temperature with TRITC-phalloidin (Sigma) and diluted to a final concentration of 0.10 μg/μl. After this, cells were washed twice for 5 minutes with PBS, once for 5 minutes with PBS containing DAPI (1:5000), and finally for 5 minutes with PBS. The cells were mounted with 50% glycerol in PBS. F-actin was visualized using epifluorescence and digital pictures were taken with a Hamamatsu C4742-95 video unit, controlled by the Openlab program (Improvision, Coventry, UK). Photoshop (Version 7.0, Adobe Systems, San Jose, CA) and Imagetrak (created by Peter K. Stys) were used for digital editing. Imagetrak was used to measure fluorescence intensity.

To visualize hemocyte patterns within larvae, wandering third-instar larvae were washed in PBS and then killed by freezing at –80°C for two minutes. The larvae were then transferred to a glass slide, covered in 50% glycerol and visualized as described previously.

Hemocyte counting and statistics

Hemocyte counting and statistics were done according to Zettervall et al. (Zettervall et al., 2004). Briefly, UAS transgenic lines were crossed to *Hemese-GAL4, UAS-GFPnls*. The females were allowed to lay eggs at 21-23°C for 2 days before the vials were moved to 29°C. Larvae were staged according the procedures described in Andres and Thummel (Andres and Thummel, 1994). Staged larvae were washed in PBS before being bled into 20 μl of PBS with a fine pair of forceps and a 27-gauge needle. The hemocyte-containing PBS was then loaded onto a improved Neubauer hemocytometer for counting. Hemocytes from at least 15 larvae of each strain were counted, and statistical analysis was carried out according the procedures outlined in Zettervall et al. (Zettervall et al., 2004).

Measurement of cell size

Hemocytes 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 hour. The cells were then stained with TRITC-phalloidin and DAPI as stated previously. F-actin was visualized using epifluorescence and digital pictures were taken with a Hamamatsu C4742-95 video unit, controlled by the Openlab program (Improvision, Coventry, UK). Cell-area measurements were made by measuring the cells on the *x* and *y* axes, using the Openlab program and taking the average of these two measurements in μm. For statistics, an initial ANOVA analysis (http://www.physics.csbsju.edu/stats/anova.html) indicated that the overexpression of the UAS constructs significantly affect hemocyte 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.

Wasp egg encapsulation assay

Encapsulation assays were done according to Sorrentino et al. (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 hours, after which the *Drosophila* larvae were transferred to apple-juice plates and left at room temperature for 40-42 hours. After this time the larvae were collected, washed in PBS and analysed under a stereomicroscope for the presence of a dark capsule. Larvae in without dark capsules were dissected in 20 μl of PBS to determine whether they had been parasitized. Larvae containing eggs of the parasitoid that had not darkened by this time were scored as non-encapsulated. Non-parasitized larvae were excluded from the count.

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