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TM9SF4 is required for Drosophila cellular immunity via cell adhesion and phagocytosis

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Summary
Nonaspanins are characterised by a large N-terminal extracellular domain and nine putative transmembrane domains. This evolutionarily conserved family comprises three members in Dictyostelium discoideum (Phg1A, Phg1B and Phg1C) and Drosophila melanogaster, and four in mammals (TM9SF1-TM9SF4), the function of which is essentially unknown. Genetic studies in Dictyostelium demonstrated that Phg1A is required for cell adhesion and phagocytosis. We created Phg1A/TM9SF4-null mutant flies and showed that they were sensitive to pathogenic Gram-negative, but not Gram-positive, bacteria. This increased sensitivity was not due to impaired Toll or Imd signalling, but rather to a defective cellular immune response. TM9SF4-null larval macrophages phagocytosed Gram-negative E. coli inefficiently, although Gram-positive S. aureus were phagocytosed normally. Mutant larvae also had a decreased wasp egg encapsulation rate, a process requiring haemocyte-dependent adhesion to parasitoids. Defective cellular immunity was coupled to morphological and adhesion defects in mutant larval haemocytes, which had an abnormal actin cytoskeleton. TM9SF4, and its closest parologue TM9SF2, were both required for bacterial internalisation in S2 cells, where they displayed partial redundancy. Our study highlights the contribution of phagocytes to host defence in an organism possessing a complex innate immune response and suggests an evolutionarily conserved function of TM9SF4 in eukaryotic phagocytes.

Key words: Innate immunity, Macrophages, Adhesion, Phagocytosis, Cytoskeleton, Nonaspanin

Introduction
Pathogen engulfment by host phagocytic cells and their subsequent killing in the phagocytic vacuole are major events for bacterial clearance and contribute to a robust innate immunity in most multicellular organisms (Beutler, 2004). In mammals, phagocytosis is mainly achieved by neutrophils, monocytes and macrophages. These cells engage additional host defences by inducing an inflammatory response, mainly through the synthesis of Rel/NF-B-dependent cytokines.

The unicellular phagocytic amoeba Dictyostelium discoideum has been used as a model organism to study and discover new genes implicated in phagocytosis (Cornillon et al., 2000). A genetic screen identified PHG1A, alteration of which causes a marked decrease in Dictyostelium adhesion to certain substrates and a strong impairment in bacterial phagocytosis and killing (Benghezal et al., 2003; Benghezal et al., 2006; Cornillon et al., 2000). PHG1A encodes a member of the TM9 protein family (also known as nonaspanins or TM9SF) characterised by the presence of nine transmembrane domains, and a high degree of evolutionary conservation (Chluba-de Tapia et al., 1997; Schimmoller et al., 1998). TM9 proteins were found in endosomal or lysosomal fractions in yeast (Singer-Kruger et al., 1993), Dictyostelium (Benghezal et al., 2003) and human cells (Bagshaw et al., 2005; Diaz et al., 1997; Schimmoller et al., 1998) where they might participate in vesicular transport (Diaz et al., 1997). More recently, TM9 proteins were implicated in lysosomal secretion in Dictyostelium and cell signalling in both Dictyostelium and yeast (Froquet et al., 2008). However, no mutant or functional data are available at the level of a metazoan organism possessing a complex immune response.

Thanks to its sophisticated immune system Drosophila represents a powerful host model for evaluating the contribution of phagocytic cells to host innate immunity. Drosophila has specialised circulating phagocytic cells derived from the haemocytic blood cell lineage (Crozatier and Meister, 2007; Williams, 2007). Plasmatocytes are the most abundant type of circulating haemocytes and represent the primary macrophages required for bacterial phagocytosis (Avet-Rochex et al., 2005; Brennan et al., 2007; Kocks et al., 2005). Upon infection by parasites, such as wasp eggs, plasmatocytes can recognise and attach to the invader. Plasmatocytes then signal to the lymph gland to promote the differentiation of another kind of haemocyte called lamellocytes (Lanot et al., 2001). These large cells attach to the plasmatocyte layer and form a hermetic capsule around the invader (Russo et al., 1996; Williams et al., 2005). In insects, plasmatocyte adhesion to wasp eggs is a crucial step for encapsulation and strongly depends on cell surface molecules such as integrins (Irving et al., 2005; Zhuang et al., 2007). Besides the cellular immune response, Drosophila possesses a sophisticated humoral response, which includes the synthesis of antimicrobial peptides by fat body cells under the control of the two

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conserved NF-κB signalling pathways Toll and Imd (immune deficiency) (Lemaitre and Hoffmann, 2007; Ferradon et al., 2007). The Imd pathway is strongly stimulated by Gram-negative bacteria resulting in the activation of the NF-κB transcription factor Relish, which in turn activates the transcription of numerous genes, in particular the antimicrobial-peptide-encoding genes Attacin (Att), Diptericin (Dipt) and Drosocin (Dro) (Georgel et al., 1993; Lemaitre et al., 1995; Levashina et al., 1998). The Toll pathway is mainly activated by fungi or Gram-positive bacteria resulting in the expression of another set of antimicrobial peptide genes including Drosomycin (Drs) (Lemaitre et al., 1995).

In this paper, we describe the molecular characterisation of the three Drosophila nonaspanins and the function in innate immunity of Phg1A/TM9SF4, the Drosophila orthologue of Dictyostelium Phg1A and human TM9SF4. We created TM9SF4-null mutant flies and showed that their sensitivity to Gram-negative bacteria was correlated to impaired haemocyte-dependent phagocytosis. TM9SF4 mutant larvae failed to properly encapsulate eggs from the avirulent wasp strain Leptopilina boulardi G486. These phenotypes are coupled to abnormal adhesion and defective cytoskeleton reorganisation in mutant plasmatocytes. Both TM9SF4 and TM9SF2, its closest parologue, were required for phagocytosis in S2 cells. Our study shows that TM9SF4 function in cell adhesion and bacterial engulfment might result from defective cytoskeleton control and that TM9SF4 plays a crucial role in cellular immunity to ensure host defence against infections.

Results

Drosophila TM9 proteins

We identified three TM9 genes in the Drosophila genome: CG7364 (chromosome 2L–34D), CG9318 (2L–38E) and CG10590 (3L–64D). Nonaspanins are divided into two subgroups presenting differential characteristic features in their N-terminal amino acid sequence (Benghezal et al., 2006; Sugasawa et al., 2001). Subgroup I is characterised by a shorter hydrophilic N-terminal sequence and a characteristic motif at position 50 (VGPYxNxQETY) whereas subgroup II contains a longer N-terminal domain (~280 amino acids) and a conserved sequence immediately after the signal peptide [FY(V/L)PG(V/L)AP] (Benghezal et al., 2003). Phylogenetic analysis revealed that CG10590 (Drosophila TM9SF3) belongs to subgroup I, along with Dictyostelium Phg1B and human TM9SF1 and TM9SF3. CG9318 (Drosophila TM9SF2) and CG7364 (Drosophila TM9SF4) share 48% identity in their amino acid sequence and belong to subgroup II, together with Dictyostelium Phg1A and human TM9SF2 and TM9SF4 (Fig. 1). The Drosophila TM9SF4 protein contains the FYVPGVAP consensus sequence at amino acid position 25 followed by nine conserved transmembrane domains; it is the closest homologue of Drosophila TM9SF2 and human TM9SF1-TM9SF4 (Fig. 1). The Drosophila TM9SF4 exhibits 46% identity with Dictyostelium Phg1A and 65% identity with human TM9SF3. This high degree of conservation suggests that the corresponding genes might share similar functions. TM9SF4 refers to Drosophila TM9SF4/Phg1A in this study.

Creating TM9SF4-knockout mutant flies

We created a Drosophila null mutant TM9SF4Δ by remobilising the P(lacW)CG7364G7245 transposon inserted into the TM9SF4 transcription unit (Fig. 2A). A 1.4 kb deletion was characterised by PCR analysis, which removed a portion of TM9SF4 coding sequences including the transcription start site and the N-terminus (Fig. 2A). Sequence analysis indicated that the surrounding genes were not affected by this deletion (data not shown). A revertant strain (Rev45) showed wild-type sequence following mobilisation and was selected as a control strain possessing similar genetic background as TM9SF4Δ. Northern blot analysis showed that no transcripts were detectable in TM9SF4Δ adult flies compared with the parental strain w1118 (Fig. 2B) or Rev45 flies (not shown). TM9SF4 is expressed at all developmental stages (Fig. 2C);
however, TM9SF4 flies are normally viable and fertile suggesting redundancy with other nonaspanins during development. Quantitative real-time PCR allowed for the detection of TM9SF4 transcripts from dissected third instar larval tissues and indicated that TM9SF4 is expressed in the main immune organs such as circulating haemocytes, fat body cells and larval gut, with the maximum expression level in haemocytes compared with the whole larvae (Fig. 2D).

TM9SF4 mutant flies have reduced resistance to Gram-negative bacteria
To assess TM9SF4 function in Drosophila resistance to bacterial infection, TM9SF4 mutant flies were infected with several bacterial species by septic injury. We reported previously that TM9SF4 (Dphg1A) mutant flies showed normal resistance to Pseudomonas aeruginosa, but reduced resistance to the Gram-negative bacteria Klebsiella pneumoniae a pathogen that was specifically not permissive for the growth of PHG1A mutant Dictyostelium (Benghezal et al., 2006). Here we show that TM9SF4 sensitivity to Klebsiella pneumoniae was not as strong as that observed for the mutant TAK1 which blocks activation of the Imd pathway (Rutschmann et al., 2000; Vidal et al., 2001) (Fig. 3A). We used a more physiological infection procedure consisting of oral infection by providing P. aeruginosa in the animal feed (Avet-Rochex et al., 2005; Avet-Rochex et al., 2007; Erickson et al., 2004; Vodovar et al., 2005). This procedure allowed detection of the significant sensitivity of TM9SF4 mutant flies compared with control Rev45 or w1118 flies, suggesting a contribution of TM9SF4 to the intestinal resistance to P. aeruginosa (Fig. 3B). In addition to K. pneumoniae and P. aeruginosa, TM9SF4 mutant flies were slightly sensitive to Gram-negative Enterobacter cloaca (Fig. 3C), whereas their resistance to nonpathogenic bacteria, such as Escherichia coli or Agrobacterium tumefaciens, was similar to that in control flies (data not shown). No difference in sensitivity was observed between TM9SF4 mutant and control flies following infection with Gram-positive Enterococcus faecalis, Staphylococcus aureus (Fig. 3D,E) or Micrococcus luteus (not shown).

To rescue TM9SF4 sensitivity to infections we constructed UAS-TM9SF4 transgenic flies allowing tissue-directed expression of the TM9SF4 cDNA by various Gal4-specific driver lines (Brand et al., 1994). However, re-expressing TM9SF4 in haemocytes either by srpGal4 (Crozatier et al. 2004), or through the more specific hmlGal4 (Goto et al., 2003) and HeGal4 (Zettavall et al., 2004) driver lines, induced pupal lethality. Vials were placed at 18°C during development, which allowed for the recovery of adults in the case of hmlGal4 (TM9SF4;hmlGal4/UAS-TM9SF4) and HeGal4 (TM9SF4;HeGal4/UAS-TM9SF4 flies) but not in the case of srpGal4 (srpGal4; TM9SF4;UAS-TM9SF4/+) flies. Surviving adult flies were placed at 25°C and infected 5 days later with K. pneumoniae. These flies expressed high levels of TM9SF4 (data not shown), but were much more sensitive than Rev45 control flies and even TM9SF4 flies, to K. pneumoniae infection (data not shown).
Increased sensitivity is probably due to the poor viability of TM9SF4-expressing flies. Indeed, TM9SF4 ectopic expression might interfere with unknown signalling pathways as suggested by the observation that tissue-directed expression of TM9SF4 induces strong morphogenesis defects (unpublished observations). Similarly, increased sensitivity was observed when Rac2 was overexpressed in the haemocyte lineage (Avet-Rochex et al., 2007). These observations indicate that expression of TM9SF4 must be finely tuned in phagocytic cells to preserve their function. Since Rev45 flies presented a wild-type phenotype (similarly to w1118 control flies) (Fig. 3A-C), the sensitivity of TM9SF4 mutant flies is unlikely to be due to a background effect. In addition, transheterozygous flies TM9SF4/Df(2L)h82a2, where the deficiency includes the TM9SF4 gene, presented a survival phenotype to K. pneumoniae infections similar to that of TM9SF4 homozygous flies (Fig. 3F).

We then observed that bacterial growth is facilitated in mutant TM9SF4 flies infected with K. pneumoniae. Indeed, bacterial numbers were greater in mutant TM9SF4 flies compared with control flies at 18 hours post infection (Fig. 3G). Therefore, sensitivity of TM9SF4-deficient flies was observed with Gram-negative pathogenic bacteria and this sensitivity is coupled to a higher bacterial growth rate.

NF-κB-dependent immune signals are not affected in TM9SF4-deficient flies

Antimicrobial peptide production by fat body cells is a major mechanism contributing to bacterial clearance following fly infection and we therefore analysed whether immune signalling was affected in TM9SF4 mutant flies. The activation of the Imd pathway was followed through the induction of Attacin, Diptericin and Drosocin (Georgel et al., 1993; Lemaitre et al., 1995; Levashina et al., 1998) and the activation of the Toll pathway, through the induction of Drosomycin (Lemaitre et al., 1996). Northern blot analysis revealed a strong induction of Attacin, Diptericin and Drosocin in TM9SF4 mutant flies, similarly to control flies, following infection with E. coli, E. cloacae or K. aerogenes (Fig. 4A, E. cloacae). As expected,
a strong inhibition of antimicrobial peptide gene expression was observed in TAK12 mutant flies (Fig. 4A). Additional quantitative real-time PCR analysis confirmed that no significant differences exist between Rev45 and TM9SF4 mutant flies in the induction of Diptericin following infection by the Gram-negative bacteria E. cloacae, K. pneumoniae (Fig. 4B) or E. coli (not shown). Similarly, the Toll pathway was activated normally in TM9SF4 mutants following infection by the Gram-positive bacteria E. faecalis (Fig. 4C) or M. luteus (Fig. 4D), resulting in the increased expression of Drosomycin. No significant changes in TM9SF4 expression were observed in flies infected with E. cloacae, K. pneumoniae or M. luteus, suggesting that TM9SF4 is not regulated at the transcriptional level by infection (data not shown).

Previous observations suggested that expression of the antimicrobial peptide encoding Defensin was particular in that it required normal haemocyte function (Brennan et al., 2007), raising the question whether TM9SF4, having defective haemocyte-dependent phagocytosis (see below), would be necessary for the induction of Defensin expression. Since genomic PCR analysis of the TM9SF4 locus was absent in this strain (data not shown), we analysed TM9SF4/Df(2L)B82a2 transheterozygous flies. These flies strongly induced Defensin expression following infection by K. pneumoniae, reaching half the level of control flies, as expected for Defensin hemizygous flies (Fig. 4E). This indicates that TM9SF4 is not required for Defensin expression. Lack of Defensin expression in TM9SF4 homozygous flies is unlikely to be the cause for increased sensitivity to Gram-negative bacteria because TM9SF4/Df(2L)B82a2 transheterozygotes showed a similar sensitivity to Gram-negative bacterial infection (Fig. 3F). In addition, Defensin is essentially active against Gram-positive bacteria in vitro (Rutschmann et al., 2002) and TM9SF4 homozygous flies resisted Gram-positive infection in the normal manner (Fig. 3D,E).

Our results demonstrate that activation of Toll and Imd immune signalling pathways by bacterial infection is not affected in TM9SF4-deficient flies, indicating that increased sensitivity of mutant flies to Gram-negative bacteria was not due to defective production of antimicrobial peptides.

**TM9SF4 is required for haemocyte-dependent phagocytosis**

In adult *Drosophila*, clusters of sessile haemocytes are present along the dorsal vessel on the anterior dorsal part of the abdomen. To assess engulfment of living bacteria by these cells, TM9SF4 mutant and Rev45 flies were injected with GFP expressing *K. pneumoniae*. Less ingested fluorescence was observed in the clustered dorsal haemocytes in TM9SF4 mutants compared with Rev45 flies at 3 hours post infection (arrowheads, Fig. 5A,B), suggesting that more bacteria escaped phagocytosis in mutant flies. Bacterial proliferation was detected in 20% of mutant flies as early as 5 hours post infection (arrows, Fig. 5D). In these flies, fluorescence was observed in the haemolymph and was also visualised in a drop of haemolymph bled from injured flies (not shown). This indicates that ingested bacteria do not multiply in phagocytic cells and that bacterial growth occurred extracellularly. In addition, haemocyte-associated fluorescence decreased both in Rev45 and TM9SF4 mutant flies (arrowheads, Fig. 5D), indicating that bacteria were, most probably, properly killed by TM9SF4 mutant haemocytes.

To quantify the phagocytosis defect of TM9SF4 mutant haemocytes, circulating plasmatocytes from TM9SF4 mutant or Rev45 third instar larvae were isolated and their ability to engulf fluorescent latex beads was observed. Mutant plasmatocytes displayed a reduced number of internalised fluorescent beads (Fig. 6A). We further measured the phagocytosis index of FITC-labelled latex beads or *E. coli* or *S. aureus*, as described previously (Avet-Rochex et al., 2005; Pearson et al., 2003). TM9SF4 plasmatocytes phagocytosed latex beads and *E. coli* two times less efficiently than wild-type cells, whereas phagocytosis of *S. aureus* was unaffected (Fig. 6B). By using the srpGal4 driver line, TM9SF4 expression was mainly induced in haemocytes (Crozatier et al., 2004) in either a Rev45 or a TM9SF4 mutant context. Rescue of the phagocytosis defect was observed in mutant plasmatocytes expressing TM9SF4 in larvae raised at 18°C (Fig. 6B). Our results indicate that TM9SF4 is required for phagocytosis of hydrophilic particles and the Gram-negative bacteria *E. coli* by plasmatocytes, whereas it is dispensable for the internalisation of the Gram-positive bacteria *S. aureus*.

**TM9SF4 is required for proper encapsulation of wasp eggs**

Cellular immunity in *Drosophila* plays a major role against bigger pathogens such as parasitoids. To elucidate whether TM9SF4 is involved in the cellular immune response against parasitisation, an encapsulation assay was performed on larvae parasitised by the avirulent *Leptopilina boulardi* wasp strain G486. When the avirulent wasp strain G486 parasitises *Drosophila* larvae a darkened cellular capsule is visible in the haemoceol 30-40 hours later. At room temperature (24°C) w1118 or Rev45 control larvae encapsulated the wasp eggs 88% and 79%, respectively, whereas only 48% of TM9SF4 mutant larval properly encapsulated and melanised foreign eggs (Fig. 6C). A stronger phenotype was observed by elevating the temperature in larvae first raised at 29°C before being parasitised. At this higher temperature, 86% of w1118 larvae and 76% of Rev45 larvae still properly encapsulated and melanised the wasp egg, yet only 13% of the homozygous TM9SF4 mutant larval properly encapsulated the egg (Fig. 6C). From this we conclude that TM9SF4 is necessary for haemocytes to properly encapsulate *L. boulardi* eggs.

**TM9SF4 mutant macrophages display defective lamellipodia and actin organisation**

The first step of phagocytosis or encapsulation requires adhesion of phagocytes to the pathogen and strong cytoskeleton...
A. Latex beads internalisation

B. Phagocytic index (% of control)

C. Encapsulation assay (% of control)

Fig. 6. TM9SF4 mutant larval haemocytes have defective phagocytosis and encapsulation. (A) Circulating plasmatocytes were isolated from third instar larvae and incubated for 15 minutes with fluorescent latex beads. The internalisation rate of FITC-latex beads was observed following addition of quenching Trypan Blue solution. (B) Using the same procedure as in A, the internalisation rate of FITC-labelled beads or E. coli or S. aureus, was calculated as the number of internalised particles per haemocyte from 300-500 haemocytes. A phagocytosis rate of 100% was attributed to control Rev45 cells in each experiment. The results are the mean ± s.d. of three independent experiments. A significant difference (Student’s t-test, P<0.03) was found in phagocytic rate for latex beads and E. coli, but not S. aureus between Rev45 and TM9SF4 mutant cells (left panel). Directed expression of TM9SF4 mainly in the haemocyte lineage through the srpGal4 driver line (srpGal4/Y; UASTM9sf4/Y:TM9sf4) partially rescued the phagocytic properties of circulating plasmatocytes (right panel) (P<0.01, Student’s t-test). (In this experiment, larvae were raised at 18°C.) (C) Encapsulation rate of control (w1118, Rev45) and mutant (TM9SF4) larvae following wasp parasitisation. The total number of parasitised larvae examined is indicated on the top of each histogram, the number in parenthesis indicates the number of larvae presenting a dark capsule. Experiments were performed at 24°C and 29°C as indicated.

rearrangements to engulf or spread on it. Circulating plasmatocytes were isolated from Drosophila Rev45 control or TM9SF4 mutant larvae, and their ability to spread on glass cover slips was studied by reflection interference contrast microscopy (RICM). Unlike phase-contrast imaging (Fig. 7A,B), RICM allows visualisation of cell-substrate contact areas, which appear dark (Gingell and Owens, 1992; Pierres et al., 2003). Control Rev45 cells displayed wild-type cell-substrate contact area morphology: they spread isotropically, and a dark ring characteristic of a lamellipodium surrounded the cells after about 15-45 minutes, indicating a close contact of the cell circumference to the substrate (Fig. 7C). By contrast, TM9SF4 plasmatocytes spread in an irregular manner and although large lamellipodium protrusions were clearly visible (Fig. 7D), the adhesive belt was absent (Fig. 7D, arrowhead) or severely disrupted. Instead, non-uniform white areas were often visible, representing portions of cells, at about 260 nm above the surface (Fig. 7D, arrow).

To further analyse their cytoskeleton organisation, circulating plasmatocytes from third instar larvae were labelled with Texas-Red-tagged phalloidin and examined by confocal microscopy. Control cells displayed a homogeneously sized surface and a round shape, as previously reported (Williams et al., 2007; Williams et al., 2006) (Fig. 7E-G). By contrast, TM9SF4 mutant cells presented heterogeneous sizes and shapes and displayed disorganised frequently long actin spikes and punctate actin accumulation (Fig. 7H-J). Quantification of the area of the actin cytoskeleton network in close contact with the surface demonstrated that mutant cells had a 2.3-fold larger average size than control Rev45 cells. This indicates that mutant cells displayed increased spreading on the substrate (Fig. 7K). Expressing TM9SF4 cDNA in mutant plasmatocytes partially reduced the extent of the cytoskeleton network, because these cells possessed a 1.34-fold larger average surface area compared with Rev45 cells (Fig. 7K). Our observations demonstrate that the nonaspanin TM9SF4 may control cell adhesion, cell shape and signalling to the actin cytoskeleton.

TM9SF4 and TM9SF2 contribute to bacterial phagocytosis in Drosophila S2 cells

Drosophila S2 cells are derived from a primary culture of late-stage embryos (Schneider, 1972). They express macrophage-specific markers, such as the two phagocytic receptors dSR-CI and Eater, and can bind and engulf particles (Kocks et al., 2005; Ramet et al., 2001). To assess functional redundancy of nonaspanins in bacterial phagocytosis, we evaluated the effect of inactivating TM9SF4 and TM9SF2, either alone or in combination. Both genes are constitutively expressed in S2 cells (data not shown) and were inactivated by silencing RNAs (siRNA) as described (Clemens et al., 2000). An equivalent reduction in phagocytosis of FITC-labelled E. coli was observed in TM9SF4- or TM9SF2-silenced cells, presenting an internalisation rate of 53% and 57%, respectively, compared with levels in wild-type cells (Fig. 8A). This phagocytosis rate was reduced to 38% when both genes were silenced. For comparison, cells treated with siRNA targeting PGRP-LC, which encodes a receptor protein required for Gram-negative bacteria internalisation by S2 cells (Ramet et al., 2002), exhibited a phagocytosis rate of FITC-labelled E. coli of 34% compared with that in control cells (Fig. 8A). No significant inhibition of FITC-labelled S. aureus internalisation was observed in TM9SF4 silenced S2 cells (Fig. 8B). By contrast, TM9SF2 silencing provoked a reduction of the phagocytosis rate of FITC-labelled S. aureus to 56% that of control cells. This inhibition was significantly increased, leading to a phagocytosis rate of 32%, when TM9SF4 was co-silenced with TM9SF2, suggesting that TM9SF4 contribution to S. aureus
engulfment was masked by redundancy with TM9SF2. Silencing Dscar, a component of the cytoskeleton required for the phagocytic process of both types of particles (Pearson et al., 2003) resulted in a strong, although not complete, reduction of phagocytosis of both FITC-labelled *E. coli* (22%) and FITC-labelled *S. aureus* (37%) (Fig. 8A,B). Our results show that the two nonaspanins TM9SF2 and TM9SF4 are both required for bacterial phagocytosis by *Drosophila* cultured cells and possess partial functional redundancy in this process.

**Discussion**

**TM9SF4 function in cell adhesion and haemocyte-dependent phagocytosis is coupled to cytoskeleton defects**

The function of the nonaspanin Phg1A in bacterial phagocytosis and cell adhesion was first demonstrated in the free-living amoeba *Dictyostelium* (Benghezal et al., 2003; Cornillon et al., 2000). Here, we show that these functions are conserved throughout evolution because DPPhg1A/TM9SF4 mutant circulating plasmatocytes isolated from *Drosophila* third instar larvae presented defective phagocytosis of latex beads and *E. coli*, two types of particles that were very poorly internalised by mutant amoeba. In addition, as observed by RICM, *Drosophila* TM9SF4 mutant macrophages have a severely disrupted adhesive belt compared with control cells, which displayed a regular lamellipodium around the entire cell circumference. In *PHG1A* mutant amoeba, no cytoskeleton defects were reported (Cornillon et al., 2000). By contrast, in *Drosophila* TM9SF4 mutant macrophages, defects in cell adhesion and phagocytosis were coupled with an abnormal actin cytoskeleton: whereas control cells presented a wild-type round shape, mutant macrophages presented disorganised actin staining and actin spikes. Interestingly similar actin spike phenotypes were described in *S2 Drosophila* cells following silencing of actin regulatory proteins such as Dscar (Kunda et al., 2003; Pearson et al., 2003). Our study thus indicates that TM9SF4 function in cell adhesion and phagocytosis might result from defective signalling in the control of actin reorganisation during cell attachment.

**TM9SF4 requirement in host cellular immunity**

When infected with pathogens, *TM9SF4* mutant flies were sensitive to the Gram-negative pathogenic bacteria but they resisted Gram-positive bacteria as well as wild-type flies did. Interestingly, *TM9SF4* mutant plasmatocytes were specifically defective for the internalisation of the Gram-negative bacteria *E. coli*, whereas no defects were observed in the internalisation of the Gram-positive bacteria *S. aureus*, putatively because of redundancy with TM9SF2 in this process (see below). Therefore, sensitivity of *TM9SF4* flies to Gram-negative bacteria might be correlated with defective phagocytosis. Supporting this hypothesis, the activation of the two NF-κB-dependent pathways, Toll and Imd, controlling the activation of the antimicrobial peptide genes by fat body cells, were not affected in *TM9SF4* mutant flies, which is consistent with the fact that these mutant flies were not as sensitive as *TAK1* mutant flies to Gram-negative pathogenic bacteria and resisted non-pathogenic strains. In an independent large siRNA screen performed in cultured *Drosophila* S2 cells, *TM9SF4* (CG7364) was also shown to be required for the internalisation of the yeast *Candida albicans* (Stroschein-Stevenson et al., 2006). In fact, we observed that *TM9SF4* mutant flies were sensitive to *C. albicans* infections (E.B.
Fig. 8. TM9SF2 and TM9SF4 are required for phagocytosis in S2 cells. S2 cells were untreated (Control) or treated for 3 days with siRNA GFP (Green fluorescent protein), TM9SF4, TM9SF2, PGRP-LC or Dscar, either alone or in combination (TM9SF2+4), as indicated. Cells were then incubated for 10 minutes with FITC-labelled E. coli (A) or FITC-labelled S. aureus (B) and the internalised fluorescence was measured in the presence of external Trypan Blue quenching solution. The phagocytosis index was quantified as the percentage of fluorescence-positive cells multiplied by the mean fluorescence of these cells. We counted 10,000-20,000 cells from each sample. A phagocytosis index of 100% was attributed to control cells. The results presented are the mean ± s.d. of three experiments. Significant differences were observed using Student’s t-test between: (A) TM9SF4 and control (P<0.003), TM9SF2 and control (P<0.02), PGRP-LC and control (P<0.001), Dscar and control (P<0.001); and (B) TM9SF2 and control (P<0.001), TM9SF2+4 and TM9SF2 (P<0.05), Dscar and control (P<0.001).

and J.P., unpublished results) suggesting that the correlation between defective phagocytosis and sensitivity to infection extends to another kind of pathogen which provokes severe infections in humans.

Finally, we showed that encapsulation of wasp eggs was partially impaired in TM9SF4 mutant larvae and that this defect was increased at elevated temperature (29°C). Since lamellocytes differentiate and are present in the haemolymph, the adhesion defects of TM9SF4-null plasmocytes might account for the reduced encapsulation rate of parasitoid eggs. Indeed, partial loss of function of the adhesion receptor β-integrin in mys mutant larvae is sufficient to induce temperature-sensitive encapsulation defects (Irving et al., 2005). Since TM9SF4 is a null allele, the temperature-sensitive phenotype of TM9SF4 mutant flies might be an indirect consequence of increased cell adhesion defects at elevated temperature, because of loss or instability of cell surface proteins, such as integrins. In amoebae, the PHG1A-null mutant also displayed a temperature-sensitive phenotype (Benghezal et al., 2003). As is the case for Dictyostelium, removing both TM9SF2 and TM9SF4 will be required in Drosophila in the future to strengthen the phenotype and further elucidate the physiological causes for phagocytosis and encapsulation defects.

TM9SF2 and TM9SF4 contribute to phagocytosis in S2 cells

TM9SF4 was also required in Drosophila S2 cells for efficient internalisation of E. coli, but not S. aureus. By contrast, TM9SF2 was required for the internalisation of both types of bacteria in these cells, indicating that TM9SF2 and TM9SF4 are only partially redundant. When compared with silencing either TM9SF2 or TM9SF4 alone, silencing both TM9SF2 and TM9SF4 resulted in a modest enhancement of the phagocytosis defect in the case of E. coli, whereas a significant enhancement was observed in the case of S. aureus. This suggests that TM9SF4 is necessary in S. aureus internalisation but that this function was masked in mutant cells by redundancy with TM9SF2. Specificity towards pathogens depends on primary events such as bacterial recognition, whereas downstream events, such as cytoskeleton reorganisation, might be equally required for every type of particle. It is thus probable that the two proteins play redundant and non-redundant functions in both the intracellular mechanisms underlying phagocytosis and the mechanisms underlying bacterial recognition, such as expression of pathogen-recognition receptors (PRRs) at the cell surface. In support of this hypothesis, it was previously established in Dictyostelium cells that TM9 proteins are most probably not recognition molecules by themselves, but contribute to the expression of cell surface proteins (Benghezal et al., 2003). Further analysis of cell surface protein expression in single and double TM9SF2- and TM9SF4-knockout cells will be required to explore the specific involvement of TM9SF2 and TM9SF4 in cell adhesion and phagocytosis.

Materials and Methods

Stocks

Flies were raised on standard culture medium at 25°C unless indicated otherwise. The following stocks were from the Drosophila Bloomington Stock Center: w;P{lacW}CG7364<0.024>Y02571 (Benghezal et al., 2003) and w;P{lacW}CG7364 k07245 or w;P{lacW}CG7364 k07245/CyO (Avet-Rochex et al., 2005). Results are expressed as percentage of surviving adult male flies 5-7 days old.

Survival rate experiments and bacterial growth in vivo

E. coli 1106, A. tumefaciens, E. cloacae, K. pneumoniae and P. aeruginosa (PAO1) were used as Gram-negative bacterial strains. S. aureus and E. faecalis and M. luteus were used as Gram-positive strains. All strains were grown on standard Luria-Broth media (LB) overnight at 37°C except A. tumefaciens (30°C). A pellet from 50 ml overnight cultures was used to infect flies, except in the case of P. aeruginosa for which a exponential phase culture diluted to OD600=0.4 was used (to reach an approximate multiplicity of infection of 50 bacteria per fly). For septic infection, 30 adult male flies 5-7 days old were pricked in the upper part of the thorax with a thin needle previously dipped into the bacterial pellet. Oral infections were conducted as described (Avet-Rochex et al., 2005). Results are expressed as percentage of surviving infected flies at different time points following infection. Experiments were repeated at least three times with results similar to those presented.

The multiplication of bacteria during Drosophila infection was quantified as described (Fauvarque et al., 2002): at each time point, 10 infected flies were harvested in Eppendorf tubes kept on ice and ground with Teflon pestle. The homogenate was suspended in LB medium (400 μl) and centrifuged for 10 minutes at 2000 rpm. Supernatants were serially diluted in LB and spread on LB plates.
Northern blotting and RT-PCR

Total RNA was extracted from adult flies using RNAiso from QiBiogene. Northern blots (15 μg RNA/lane) were probed with a 32P-labelled fragment of TM9SF4 cDNA (2619 bp) or Dptiercin (1000 bp) or Drosophilin (376 bp), and with actin as an internal loading control (1239 bp). For the RT-PCR, total RNA were similarly extracted either from a pool of 10^5 S2 cells or from circulating plasmatocytes isolated from eight third instar larvae. One μg of total RNAs were submitted to a DNase digestion step [DNA-free TM kit (Ambion, Inc.)] and the amplification was performed according to the manufacturer’s instructions [Titanium One-Step RT-PCR kit (BD Biosciences)]. Primers were as follows: TM9SF4 forward, 5’-GAGGTTGCGAATAGAACGCT-3’; reverse, 5’-CTGGACATCCGC- CATGGCAAAGTCG-3’. Actin forward, 5’-ACCGGGTACGGTAGTCTTCTA-3’; reverse, 5’-TATGTTTGTTCATGCTGCTGTA-3’. Controls were run with no reverse transcriptase. Amplified cDNA were either visualised by ethidium bromide staining on agarose gel, or transferred onto nitrocellulose by Southern blot and probed with a reverse transcriptase. Amplified cDNA were either visualised by ethidium bromide staining on agarose gel, or transferred onto nitrocellulose by Southern blot and probed with a TM9SF4-specific probe.

For real-time PCR analysis, cDNAs were either visualised with AffinityScript QPCR cDNA Synthesis Kit (Stratagene). An amount of cDNA equivalent to 500 ng were normalised to the 35S actin gene.

Cell culture and FACS analysis

Drosophila S2 cells were maintained in Schneider’s Drosophila medium supplemented with 10% heat-inactivated FCS (all from Invitrogen). Gene inactivation was obtained by incubating 15 μg double strand RNA (RNAs) for 72 hours at 26°C with 10^5 S2 cells cultured in six-well tissue culture plates (Clemens et al., 2000). DNA templates were generated by PCR using the primers specific for each target gene: Docar forwar, 5’-taataaactcatatgggcatgggccgcaacggttgatcagc3’; reverse, 5’-taataaactcatatatggccgcaacggttgatcagc3’. GFP forward, 5’-taataaactcatatatggccgcaacggttgatcagc3’. GFP reverse, 5’-taataaactcatatatggccgcaacggttgatcagc3’. TM9SF4 forward, 5’-taataaactcatatatggccgcaacggttgatcagc3’. TM9SF4 reverse, 5’-taataaactcatatatggccgcaacggttgatcagc3’. TM9SF4 reverse, 5’-taataaactcatatatggccgcaacggttgatcagc3’. TM9SF4 reverse, 5’-taataaactcatatatggccgcaacggttgatcagc3’.

Purified PCR products were used as DNA templates for in vitro transcription of RNA using the MEGAscript RNAi kit (Ambion). Flow-cytometry-based phagocytosis assays were performed as described (Ramet et al., 2002). Briefly, S2 cells were incubated for 10 minutes at 29°C with FITC-labelled particles and flow cytometry was used to analyse the internal fluorescence rate produced by internalised L. edwardsi (K-12 strain) BioParticles FITC and S. aureus (Wood strain without protein A) BioParticles FITC (Molecular Probes). The samples were analysed using the CELLQuest program (Becton Dickinson).

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